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(71) Applicant: UNIVERSITY PATENTS, INC. [US/US]; P.O. Box 901, Westport, CT 06881 (US).

(72) Inventors: CARUTHERS, Marvin ; 2450 Cragmoor, Boulder, CO 80303 (US). BRILL, Wolfgang ; 1138 Grand View Avenue, Boulder, CO 80302 (US). NIELSEN, John ; Skyttehaven 2 - 11B, DK-2950 Vedbaek (DK).

(74) Agent: YAHWAK, George, M.; University Patents, Inc., P.O. Box 915, Westport, CT 06881 (US).

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(54) Title: NUCLEOSIDE AND POLYNUCLEOTIDE THIOPHOSPHORAMIDITE AND PHOSPHORODITHIOATE COMPOUNDS AND PROCESSES

(57) Abstract

The present invention relates to new and useful nucleoside thiophosphoramidite, polynucleotide dithioate phosphoramidite and polynucleotide phosphorthioamidate phosphoramidite compounds as well as the processes whereby these compounds can be used for synthesizing new mononucleotides and polynucleotides having phosphorodithioate, phosphorothioamidate, phosphorothiotriesters, and phosphorothioate internucleotide linkages.

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Nucleoside and Polynucleotide Thiophosphoramidite and Phosphorodithioate Compounds and Processes

Research leading to the making of the invention described herein was supported, in part, with federal funds. Accordingly, the United Stated Government has certain statutory rights to the invention described herein.

This is a continuation-in-part application of our earlier filed United States Patent Application 198,886, filed 26 May 1988.

This invention relates to new and useful phosphorus compounds which are particularly useful in the production of polynucleotides having analogs attached to phosphorus.

The present invention relates to new and useful nucleoside thiophosphoramidite, polynucleotide dithioate phosphoramidite and polynucleotide phosphorthioamidate phosphoramidite compounds as well as the processes whereby these compounds can be used for synthesizing new mononucleotides and polynucleotides having phosphorodithioate, phosphorothioamidate, phosphorothiotriesters, and phosphorothioate internucleotide linkages. These new mononucleotides and oligonucleotides can be used for many biological, therapeutic, and diagnostic applications. Potential therapeutic applications include treating tumors, viral infections and bacterial infections. Additionally, these compounds can be used to deliver metal ions, toxins, intercalating agents and other reagents that alter the biochemical reactivity of polynucleotides and proteins to specific sites in cells and tissues. These compounds can also be used for diagnostic purposes. By attaching fluorescent or other chemically reactive reagents, antigens, antibodies, proteins, and metal ions to

these compounds, they can be used for diagnosing diseases and the normal and abnormal biochemistry of cells, tissues, and body fluids such as blood and urine. There are also many uses in modern biology and chemistry as well. For example, these compounds can be used to develop improved methods for sequencing and cutting DNA, for imaging in X-ray crystallography, NMR, and electron microscopy, and for studying enzymic reactions.

High yielding methodologies are currently available for the rapid synthesis of sequence defined polynucleotides having the natural internucleotide linkage (M. H. Caruthers, Science 230, 281-285, 1985; M. H. Caruthers and S. L. Beaucage, U.S. Patent 4,415,732; M. H. Caruthers and M. D. Matteucci, U.S. Patent 4,458,066). An important step in this process is oxidation of the intermediate phosphite triester to the naturally occurring phosphate triester with aqueous iodine. These phosphite triesters can also be oxidized under anhydrous conditions with amines or ammonia and iodine to yield variable reported amounts of phosphoramidates or with sulfur to yield phosphorothioates (B. Uznanski, M. Koziolkiewicz, W. J. Stec, G. Zon, K. Shinozuka, and L. Marzili, Chemica Scripta 26, 221,224, 1986; M. J. Nemer and K. K. Ogilvie, Tetrahedron Lett. 21, 4149-4152, 1980). Other methods employing H-phosphonate internucleotide linkages can also be used to synthesize phosphoramidates (B. C. Froehler, Tetrahedron Lett. 27, 5565-5568, 1986). Unfortunately, none of these procedures can be used to synthesize polynucleotides containing the phosphorodithioate or the phosphorothioamidate internucleotide linkages.

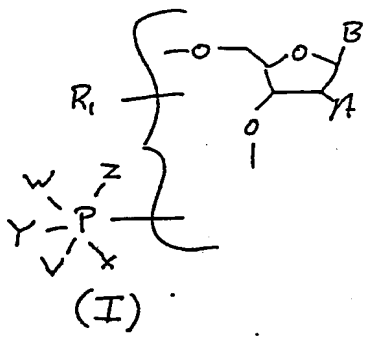
The production of uridine 2',3'-cyclic phosphorodithioate is described in the literature (F. Eckstein, J. Am. Chem. Soc. 92, 4718-4732, 1970). Unfortunately, the process cannot be used to

synthesize deoxynucleoside phosphorodithioates or nucleoside phosphorodithioates useful for synthesizing polynucleotides containing the dithioate linkage. The procedure also yields a mixture of mononucleotides having phosphorodithioate and phosphorothioate moieties. Additionally the yield of uridine 2',3'-cyclic phosphorodithioate is only 28% and the acidity of P_2S_5 and the high temperatures used in the synthesis of the cyclic phosphorodithioate would preclude the use of this procedure with protected deoxyadenosine which would undergo depurination.

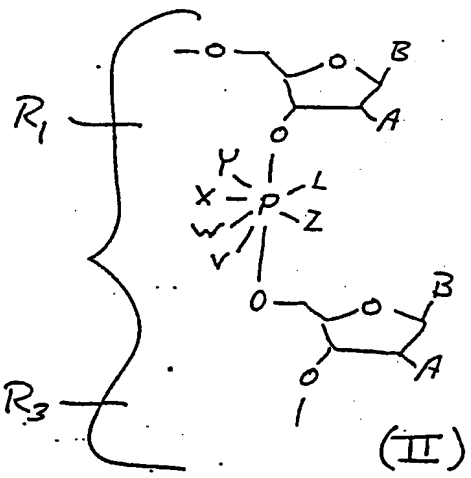
Similarly adenosine cyclic 3',5'-phosphorodithioate can be synthesized by treating suitably protected adenosine with 4-nitrophenylphosphoranilidochloridothioate followed by cyclization with potassium t-butoxide and conversion to the dithioate in a reaction with sodium hydride/carbondisulfide (J. Boraniak and W. Stec, J. Chem. Soc. Trans. I, 1645,1987). Unfortunately these reaction conditions and the low synthesis yields preclude the use of this chemistry for synthesizing oligonucleotides having phosphorodithioate linkages.

The present invention provides new and useful nucleotides, dinucleotides and polynucleotides having structure modifications at phosphorus. It also describes processes which for the first time lead to the synthesis of these compounds.

In general, the compounds, according to the present invention, can be represented by general formulae I and II:



(I)



(II)

where R_1 is H or a blocking group; P (L, V, W, X, Y, Z) is a phosphorus derivative such that L, V, W, X, Y, Z are substituents where heteroatoms are linked covalently to phosphorus; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; B is a nucleoside or deoxynucleoside base; R_3 is H or a blocking group. Substituents V,W, X and Y may also be covalently linked to heteroatom substituted or unsubstituted alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, alkenyl, cyloalkenyl, aralkenyl, alkynyl, aralkynyl, or cycloalkynyl groups.

The compounds of general formulae I and II wherein L, V, W, X, Y and Z are substituents where heteroatoms are linked to phosphorus include those in which the heteroatoms are sulfur, nitrogen and oxygen. The substituent V is oxygen single bonded to phosphorus and to either H or R_4 where R_4 is a heteroatom substituted or unsubstituted alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, alkenyl, aralkenyl, cycloalkenyl, alkynyl, aralkynyl or cycloalkynyl group. The substituent Y is sulfur single bonded to phosphorus and to either H or R_5 where R_5 is a heteroatom substituted or unsubstituted alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, alkenyl, aralkenyl, cycloalkenyl, alkynyl, aralkynyl or cycloalkynyl. The substituents W and X are nitrogen heteroatoms where W is primary amino, NHR_6, and X is secondary amino, NR_6R_7, groups. The R_6 and R_7 groups taken separately each represent heteroatom substituted or unsubstituted alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, aralkenyl, cycloalkenyl, alkenyl, aralkynyl, cycloalkynyl, or alkynyl groups. R_6 and R_7 when taken together form an alkylene chain containing up to 5 carbon atoms in the principal chain and a total of up to 10 carbon atoms with both

terminal valence bonds of said chain being attached to the nitrogen atom to which R_6 and R_7 are attached; and R_6 and R_7 when taken together with the nitrogen atom to which they are attached may also form a nitrogen heterocycle including at least one additional heteroatom from the group consisting of nitrogen, oxygen or sulfur.

The new compounds of general formula I are of two classes, Ia and Ib; class Ia consists of those in which phosphorus is single bonded through the heteroatoms to each of two substituents, X and Y where Y is attached to R_5; and class Ib are those in which Z is sulfur double bonded to phosphorus plus two other substituents from the group V and Y where the heteroatom of each of these substituents is single bonded to phosphorus. Compounds in class Ia are useful for synthesizing polynucleotides containing phosphorodithioate and phosphorothioate internucleotide linkages. Compounds in class Ib are useful for various therapeutic and biological studies and as intermediates for synthesizing nucleotides having phosphorodithioate moieties.

Compounds of general formula II are those in which all compounds have phosphorus double bonded to Z or L and single bonded to one of the substituents V, X, Y or W. Compound II is preferably phosphorus double bonded to sulfur and single bonded to Y, V, W or X. Compounds of general formula II may also be those in which L is oxygen double bonded to phosphorus plus Y which is single bonded to phosphorus. Compounds II are useful for various therapeutic, diagnostic, and biological studies and for synthesizing polynucleotides containing phosphorodithioate, phosphorothioamidate, phosphorothioate triester and phosphorothioate and phosphorodithioate internucleotide linkages which are also useful as therapeutic, diagnostic, or research reagents.

As used herein the symbols for nucleotides and polynucleotides are according to the IUPAC-IUB Commission of Biochemical Nomenclature Recommendations (Biochemistry 9, 4022, 1970). Several chemical terms as used in this invention are further defined as follows: These definitions apply unless, in special cases, these terms are defined differently.

alkyl - a non-cyclic branched or unbranched hydrocarbon radical having from 1 to 20 (preferably 1 to 12) carbon atoms. Heteroatoms, preferably oxygen, sulfur, or nitrogen, can replace carbon atoms, preferably 1 to 4 carbon atoms (or bonded to the carbon atoms) in this non-cyclic branched or unbranched radical.

aryl - an organic radical derived from an aromatic hydrocarbon by the removal of one hydrogen atom. This radical can contain one or more heteroatoms as part of the aromatic hydrocarbon ring system.

aralkyl - an organic radical in which one or more aryl groups, preferably 1 to 3, are substituted for hydrogen atoms of an alkyl radical.

cycloalkyl - a cyclic hydrocarbon radical containing from 3 to 20 (preferably 3 to 12) carbons; heteroatoms, preferably oxygen, sulfur, and nitrogen, can replace (or be bonded to) the atoms in this cyclic hydrocarbon radical.

cycloalkylalkyl - an organic radical in which one or more cycloalkyl radicals, preferably 1 to 3, are substituted for hydrogen atoms of an alkyl radical containing from 1 to 20 carbon atoms, preferably 1 to 12 carbon atoms.

alkenyl - an aliphatic, unsaturated, branched or unbranched hydrocarbon having at least one double bond and 2 to 20 (preferably 3 to 10) carbons. Heteroatoms, preferably sulfur, oxygen, and nitrogen, can replace saturated carbon atoms in this radical or be bonded to the saturated carbon atoms.

aralkenyl - an organic radical with one or more aryl groups, preferably 1 to 3, are substituted for hydrogen atoms of an alkenyl radical.

cycloalkenyl - a cyclic hydrocarbon radical having from 3 to 20 (preferably 4 to 12) carbons, and at least one double bond. Heteroatoms, preferably oxygen, sulfur and nitrogen, can replace saturated carbons in this radical or be bonded to the saturated carbons.

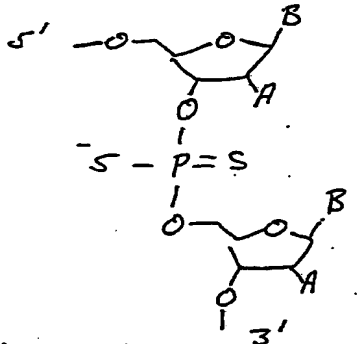
alkynyl - an aliphatic, unsaturated, branched or unbranched hydrocarbon radical containing at least one triple bond and 2 to 20 (preferably 3 to 10) carbons. Heteroatoms, preferably oxygen, sulfur, and nitrogen, can replace (or be bonded to) saturated carbons in this radical.

aralkynyl - an organic radical in which one or more aryl groups, preferably 1 to 3, are substituted for hydrogen atoms of an alkynyl radical.

cycloalkynyl - a cyclic hydrocarbon radical containing from 6 to 20 carbon atoms, preferably 7 to 12 carbon atoms, and at least one triple bond. Heteroatoms, preferably oxygen, sulfur and nitrogen, can replace saturated carbon atoms in this radical.

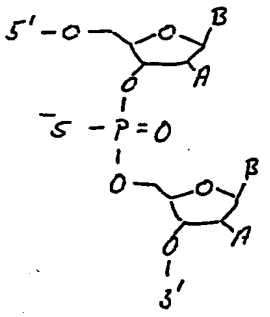
Heteroatom substituted radicals - In all these radicals, including alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, alkenyl, aralkenyl, cycloalkenyl, alkynyl, aralkynyl, and cycloalkynyl, heteroatoms, preferably sulfur, oxygen, nitrogen, and halogens, can replace hydrogen atoms attached to carbon. As described in the definition for each radical, heteroatoms, preferably oxygen, sulfur and nitrogen, can replace carbon atoms at saturated positions in alkyl, aralkyl, cycloalkyl, cycloalkylalkyl, alkenyl, aralkenyl, cycloalkenyl, alkynyl, oralkynyl, and cycloalkynyl radicals. Heteroatoms, preferably sulfur, oxygen, and nitrogen can also replace carbon as part of the aromatic ring system in aryl radicals. Of course, heteroatoms cannot replace carbon atoms in a radical where the carbon atom to be replaced is joined to the heteroatom linked to phosphorus.

phosphorodithioate internucleotide linkage - an internucleotide linkage having the general formula 5'-nucleoside-O-PS_2-O-nucleoside-3' which can be illustrated with the following structure where B and A are as defined previously.

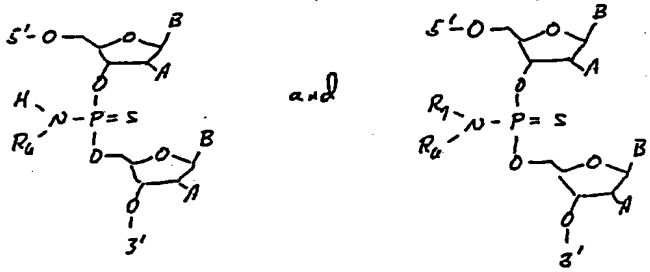


phosphorothioate internucleotide linkage - an internucleotide linkage having the general formula 5'-nucleoside-O-POS-O-nucleoside-3'

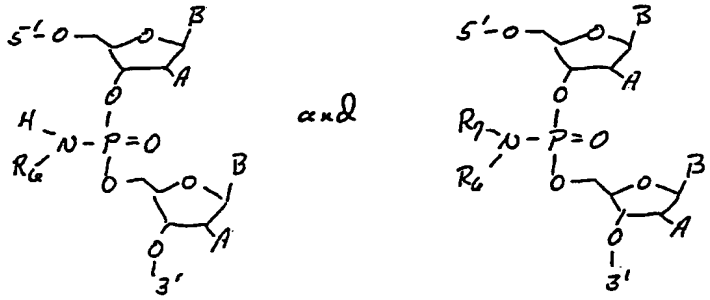
which can be illustratred with the following structure where B and A are as defined previously.



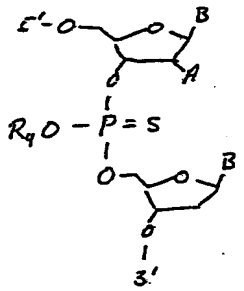
phosphorothioamidate internucleotide linkage - an internucleotide linkage having the general formula 5'-nucleoside-O-PSNHR$_6$-O-nucleoside-3' and 5'-nucleoside-O-PSNR$_6$R$_7$-O-nucleoside-3' which can be illustrated with the following structures where B,A,R$_6$ and R$_7$ are as previously defined.



phosphoromidate internucleotide linkage - an internucleotide linkage having the general formulae 5'-nucleoside-O-PONHR$_6$-O-nucleoside-3' and 5'-nucleoside-O-PONR$_6$R$_7$-O-nucleoside-3' which can be illustrated with the following structures where B,A,R$_6$ and R$_7$ are as previously defined.



phosphorothiotriester internucleotide linkage - an internucleotide linkage having the general formulae 5'-nucleoside-O-$PSOR_4$-O-nucleoside-3' which can be illustrated with the following structure where B,A, and R_4 are as previously defined.



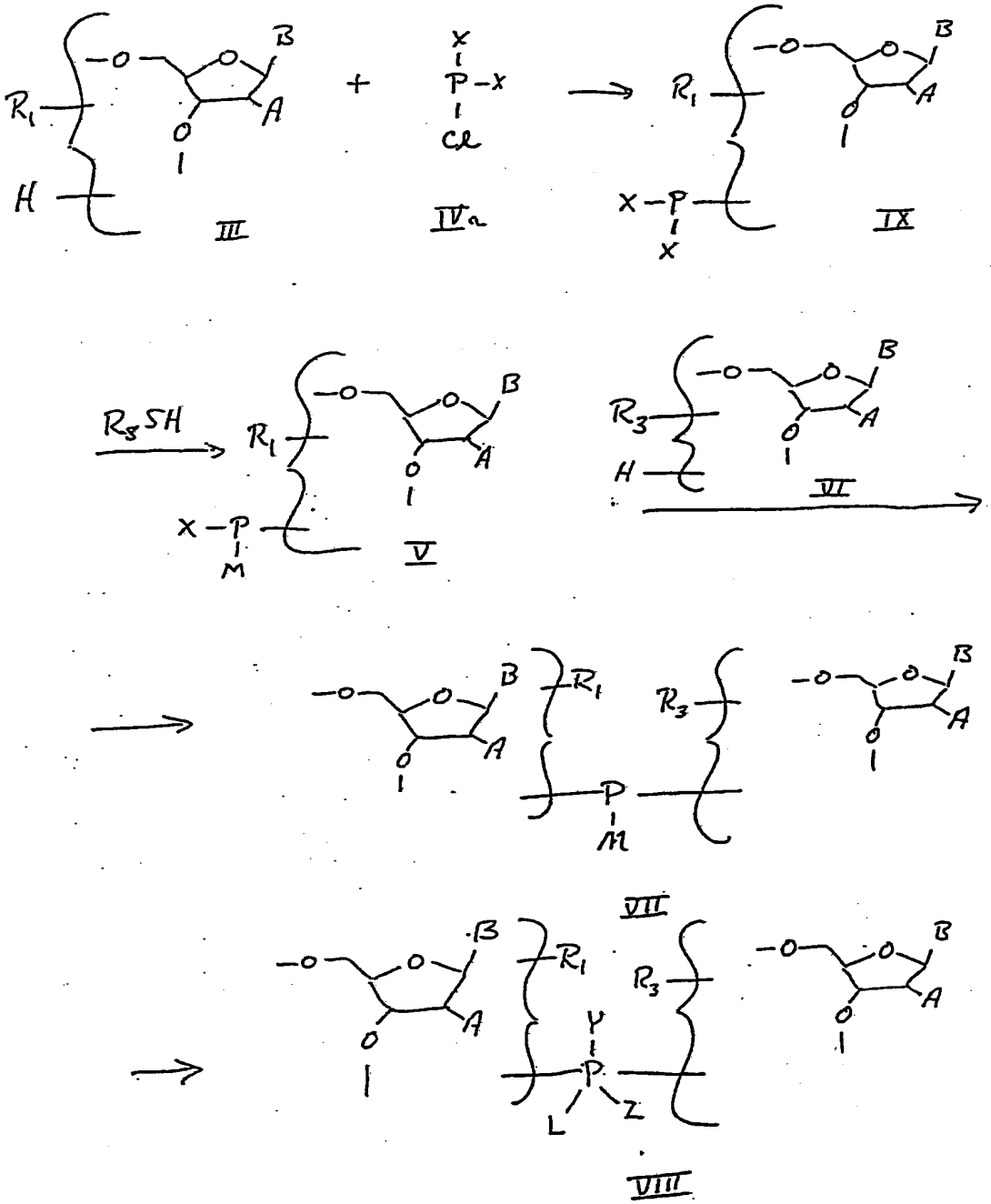
Amines from which the substituent group W can be derived include a wide variety of primary amines such as methylamine, ethylamine, propylamine, isopropylamine, aniline, cyclohexylamine, benzylamine, polycyclic amines containing up to 20 carbons, heteroatom substituted aryl or alkylamines having up to ten heteroatoms, preferably oxygen, sulfur nitrogen or halogen, and similar primary amines containing up to 20 carbon atoms. Amines from which the substituent group X can be derived include a wide variety of secondary amines such as dimethylamine, diethylamine, diisopropylamine, dibutylamine, methylpropylamine, methylhexylamine, methylcyclopropylamine, ethylcyclohexylamine, methylbenzylamine, methylcyclohexylmethylamine, butylcyclohexylamine, morpholine, thiomorpholine, pyrrolidine, piperidine, 2,6-dimethylpiperidine, piperazine, and heteroatom substituted alkyl or aryl secondary amines containing up to 20 carbon atoms and ten heteroatoms from the group consisting of sulfur, oxygen, nitrogen and halogens.

The nucleoside and deoxynucleoside bases represented by B in the above formulae are well known and include purines, e.g., adenine, hypoxanthine, guanine, and their derivatives, and pyrimidines,

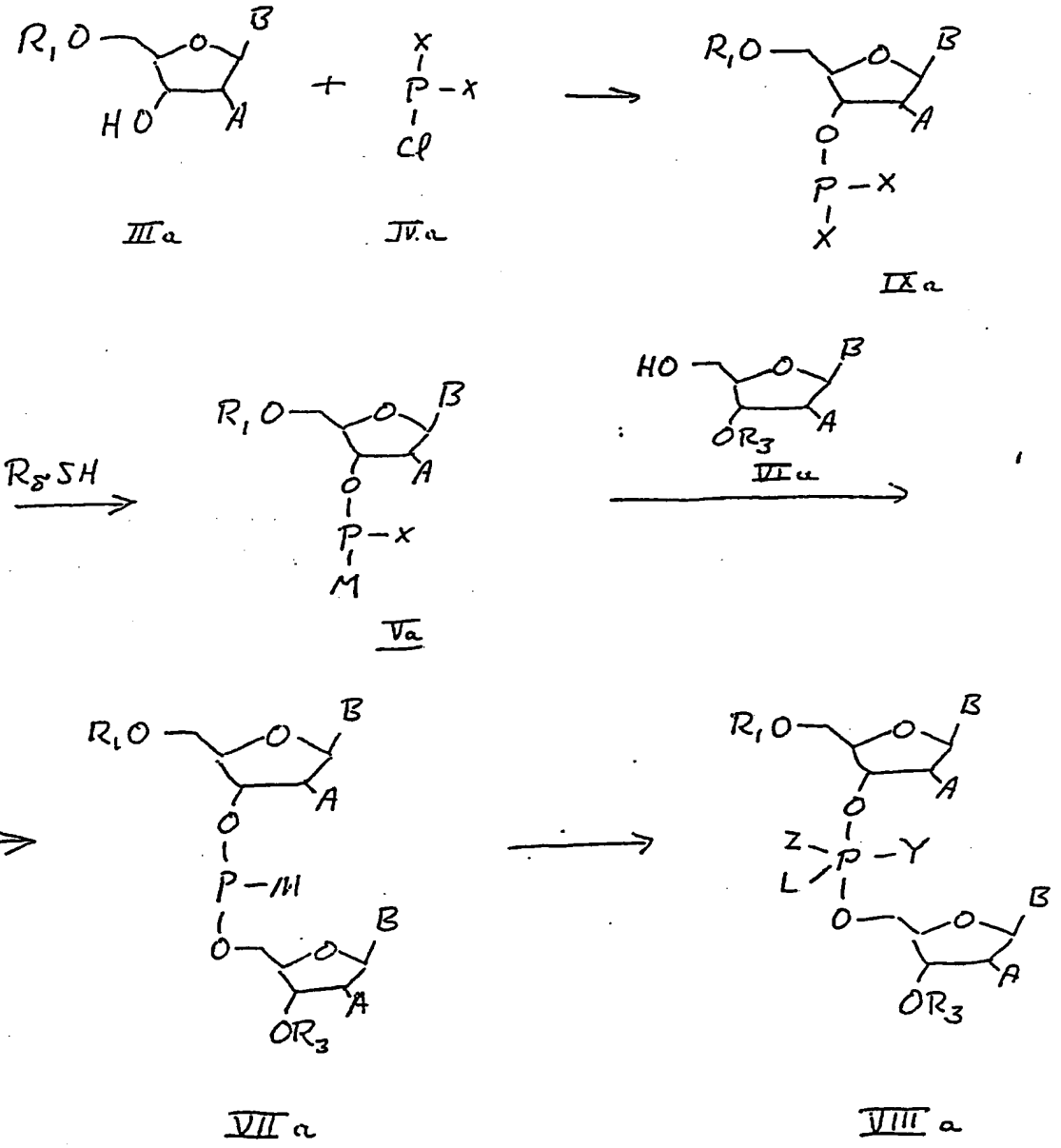
e.g., cytosine, uracil, thymine, and their derivatives.

The blocking groups represented by R_1, R_2 and R_3 in the above formulae include triphenylmethyl (trityl), p-anisyldiphenylmethyl (methoxytrityl), di-p-anisylphenylmethyl (dimethoxytrityl), pivalyl, acetyl, 4-methoxytetrahydropyran-4-yl, tetrahydropyranyl, phenoxyacetyl, isobutyloxycarbonyl, t-butyldimethylsilyl, triisopropylsilyl, alkyl or aryl carbonoyl, and similar blocking groups well known in the art.

The general reaction scheme A for synthesizing compound Ia and II is shown in the following overview:


III
IVa
IX
V
VI
VII
VIII

The preferred reaction scheme A is represented as follows:



wherein R_1, R_3, B, A, X, Z, L and Y are as previously defined; and M is sulfur single bonded to phosphorus and to R_8 where R_8 is a heteroatom substituted or unsubstituted alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, alkenyl, cycloalkenyl, aralkenyl, alkynyl, aralkynyl or cycloalkynyl. Compounds VIII and VIIIa are those in which phosphorus is linked through a single bond to Y and through a double bond to Z or L. Thus, it can be seen that compounds VIII and VIIIa are a subset of compounds II. Likewise, compounds V and Va are a subset of compounds Ia.

The process of reaction scheme A involves condensation of IIIa with IVa, which preferably is bis(dimethylamino)chlorophosphine or dipyrrolidinylchlorophosphine, to yield IXa in the presence of triethylamine. Further addition of a mercaptan, which preferably is 2,4-dichlorobenzylmercaptan, in the presence of triethylamine hydrochloride generated in the first step leads to the conversion of IXa to Va. Table 1 lists the ^{31}P-NMR characterization data for a series of Va derivatives where the nucleoside base (B), amine functionality (X), and mercaptan (M) are altered in a systematic manner. Reaction of Va with VIa and an activator (e.g., tetrazole, 5-substituted tetrazoles and substituted triazoles, alkylammonium salts, arylalkylammonium salts, substituted and unsubstituted pyridinium salts of tetrafluoroborate, and substituted and unsubstituted pyridinium and imidazolium salts of acids, 5-substituted tetrazoles, halogenated carboxylic acids and N-hydroxybenzotriazole) yields VIIa, the dinucleoside S-(2,4-dichlorobenzyl) phosphite, which can be preferably oxidized with sulfur to yield VIIIa, the dinucleoside phosphorodithioate triester with P(Y,Z). Of course oxidation with t-butylperoxide yields the

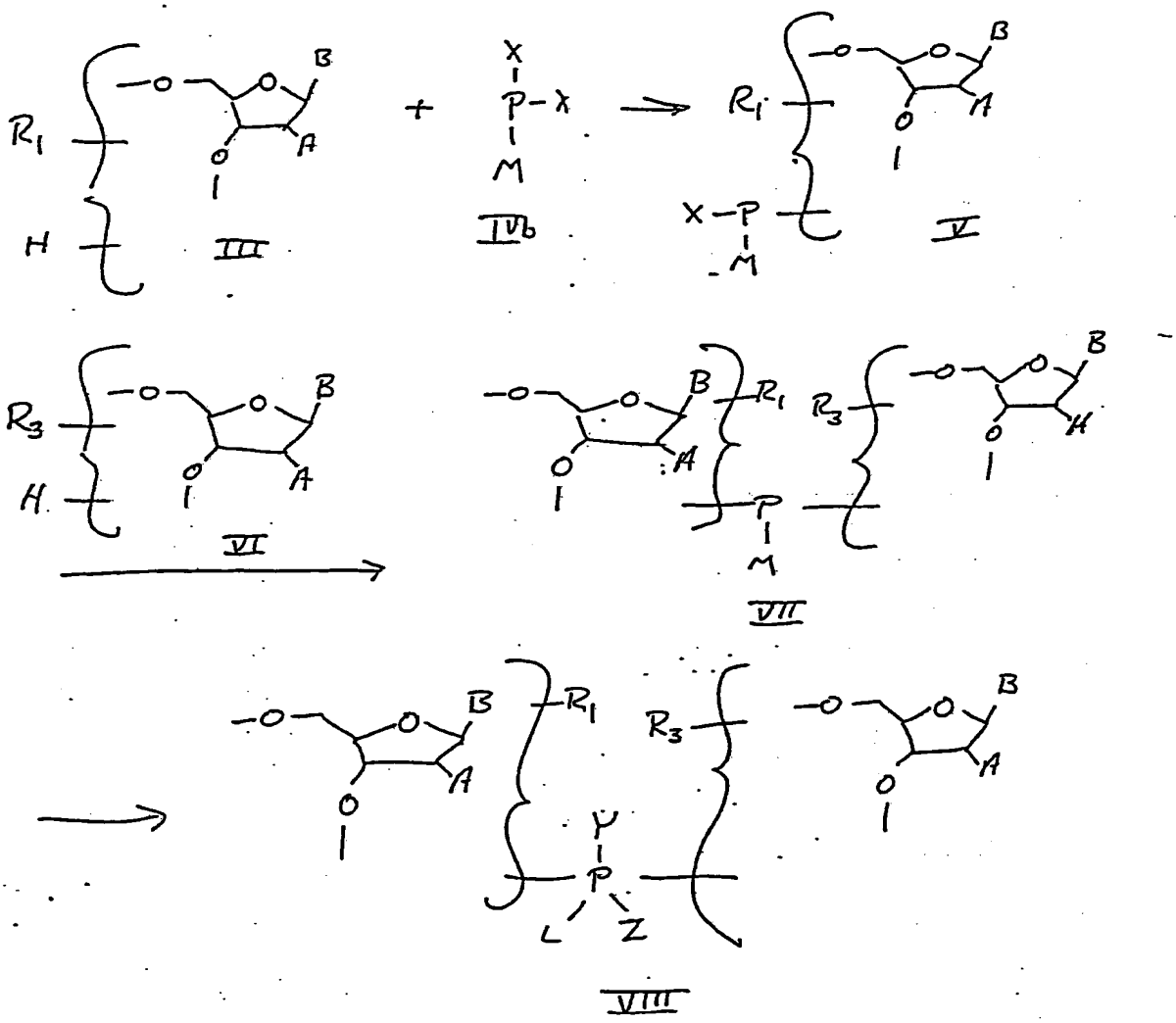
corresponding dinucleoside phosphorothioate triester P(Y,L).

Table 1. ^{31}P-NMR Characterization of Deoxynucleoside Phosphorothioamidites (Va)

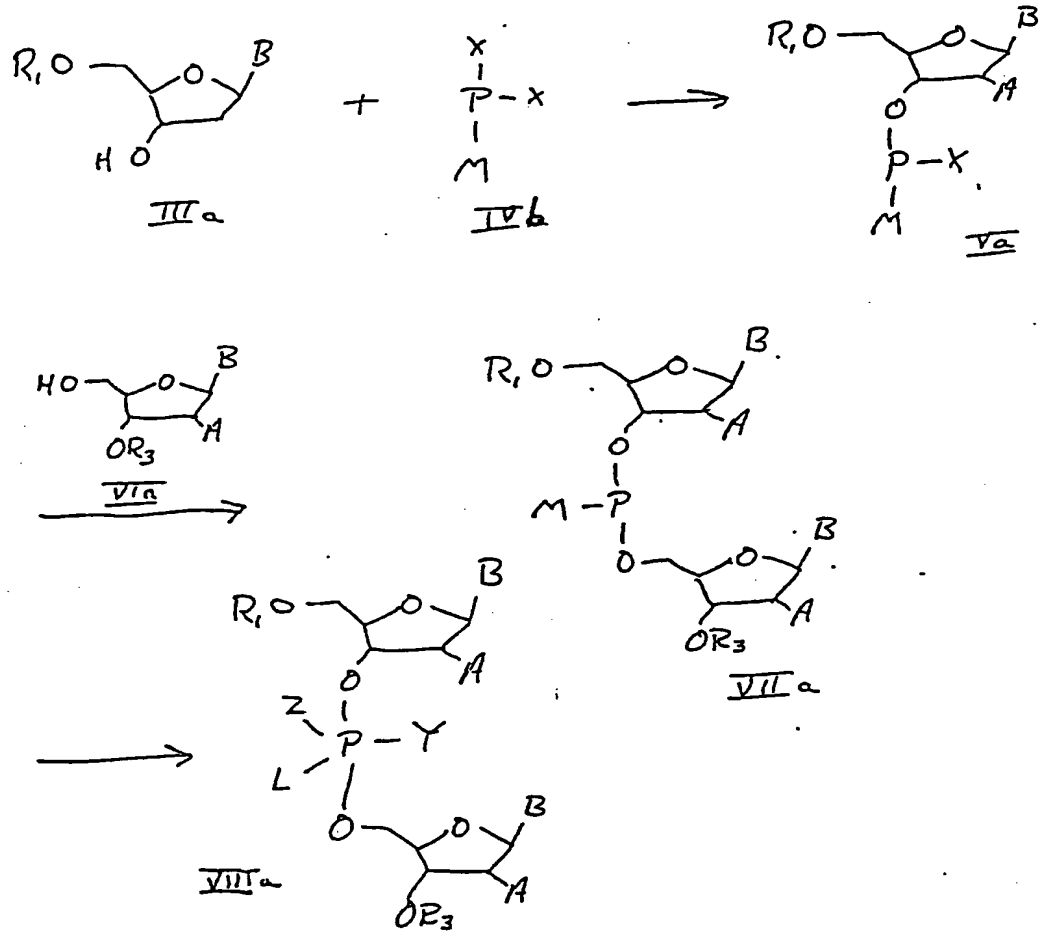
Base (B)	Amine (X)	Mercaptan (M)	^{31}p-NMR[a] (δ)
T	pyrrolidinyl	2,4-dichlorobenzyl	164.8;161.8
T	pyrrolidinyl	4-chlorobenzyl	164.2;161.0
T	dimethylamino	4-chlorobenzyl	172.3;170.5
T	dimethylamino	2,4-dichlorobenzyl	172.1;170.4
C^{Bz}	pyrrolidinyl	2,4-dichlorobenzyl	165.1;162.6
C^{Bz}	pyrrolidinyl	4-chlorobenzyl	161.8;159.9
C^{Bz}	dimethylamino	4-chlorobenzyl	171.9;170.7
C^{Bz}	dimethylamino	2,4-dichlorobenzyl	172.0;171.0
A^{Bz}	pyrrolidinyl	2,4-dichlorobenzyl	163.8;162.7
A^{Bz}	pyrrolidinyl	4-chlorobenzyl	163.5;162.3
A^{Bz}	dimethylamino	4-chlorobenzyl	171.8;170.9
A^{Bz}	dimethylamino	2,4-dichlorobenzyl	171.7;170.9
G^{iB}	pyrrolidinyl	2,4-dichlorobenzyl	163.9;160.9
G^{iB}	pyrrolidinyl	4-chlorobenzyl	163.4;161.6
G^{iB}	dimethylamino	4-chlorobenzyl	171.5;169.5
G^{iB}	dimethylamino	2,4-dichlorobenzyl	171.9;169.6

a ^{31}P-NMR were recorded in $CDCl_3$ on a Brucker WM-250 with 85% aqueous H_3PO_4 as external standard. T, C^{Bz}, A^{Bz}, and G^{iB} refer to thymine, N-benzoylcytosine, N-benzoyladenine, and N-isobutyrylguanine respectively; R_1 is dimethoxytrityl; A is hydrogen.

A second general reaction scheme for synthesizing compounds Ia and II, scheme B, is shown in the following overview:


III
IVb
V
VI
VII
VIII

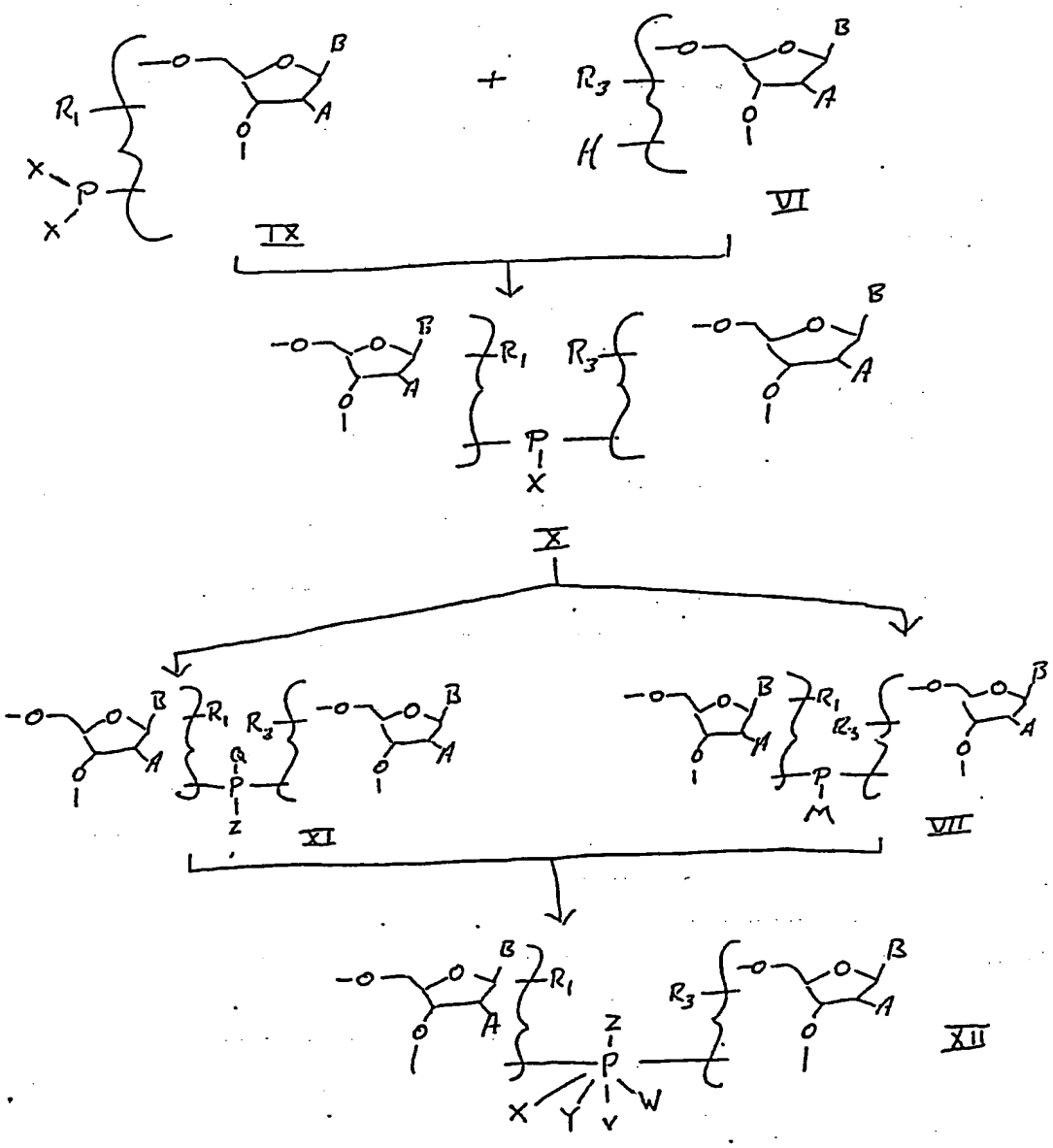
The preferred reaction scheme B is represented as follows:



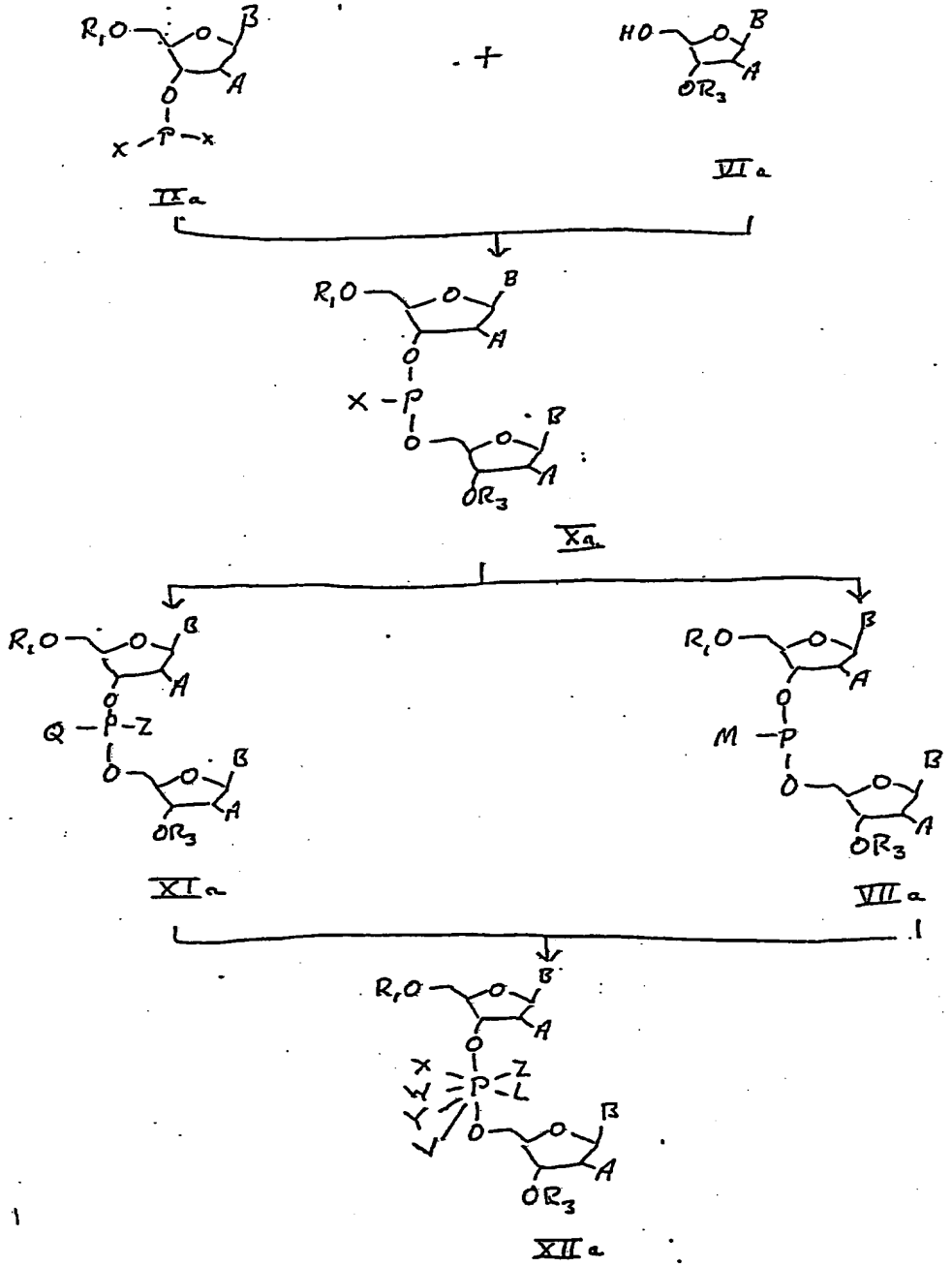
Thus it can be seen that the processes of reaction schemes A and B are identical except for the use of two different reagents, IVa or IVb, in order to generate V and Va. Reagent IVa is a bis (secondary amino) chlorophosphine whereas IVb is a bis (secondary amino) mercaptylphosphine. The use of IVa is a more general reaction leading to V and Va as these bis (secondary amino) chlorophosphines are more easily purified by distillation. Of course the use of IVa generates an intermediate diamidite, IX and IXa, to which the mercaptan is added to form V and Va. The use of IVb, leading directly to V and Va, is restricted to compounds IVb where the thiodiamidite can be purified by crystallization or distillation without decomposition. The process of reaction scheme B involves condensation of IIIa with IVb which is 4-chlorobenzylmercaptyl-bis(diisopropylamino)phosphine to yield Va with tetrazole as catalyst. Reaction of Va with VIa and an activator (e.g., 5-substituted tetrazoles and substituted triazoles, alkylammonium salts, arylalkylammonium salts, substituted and unsubstituted pyridinium salts of tetrafluoroborate, and substituted and unsubstituted pyridinium and imidazolium salts of acids, 5-substituted tetrazoles, halogenated carboxylic acids and N-hydroxybenzotriazole) yields VIIa, the dinucleoside S-(4-chlorobenzyl)phosphite, which can be preferably oxidized with sulfur to yield VIIIa, the dinucleoside phosphorodithioate triester with P(Y, Z). Of course oxidation with t-butylperoxide yields the corresponding dinucleoside phosphorothioate triester, P (Y, L). Activators that are more acidic than tetrazole, such as certain 5-substituted tetrazoles (e.g. 5-(*p*-nitrophenyl)tetrazole) and pyridinium tetrafluoroborate, can be used with success to

activate Va. Certain side reactions, however, can lead to reductions in yields of the correct product.

A third reaction scheme, scheme C, was also discovered for the purpose of synthesizing compound II. The general reaction scheme C for synthesizing Compound II is as follows:



The preferred reaction scheme C is represented as follows:



wherein R, R_3, B, A, X, W, Z, Y, and V are as previously defined and Q is H. Compounds XII and XIIa are those in which Z is sulfur double bonded to phosphorus plus one other substituent from the group of substituents V, W X and Y which are single bonded to phosphorus. These are derived from XI or XIa. Compounds XII and XIIa can also be L which is oxygen double bonded to phosphorus plus Y which is single bonded to phosphorus. These are derived from XI or XIa.

The process of reaction scheme C involves synthesis of IXa from a protected nucleoside and a bis (secondary amino) chlorophosphine and then condensation with VIa to yield Xa. Reaction of Xa with H_2S and an activator such as tetrazole yields the dinucleoside H-phosphonothioate, XIa, which can be chemically converted by oxidation with sulfur to dinucleoside phosphorodithioates, P (Z,Y); by oxidation with iodine in the presence of amines to phosphorothioamidates, P (Z, W or X); by alkylation of the corresponding dinucleoside phosphorodithioate to phosphorodithioate triesters, P (Z,Y); by oxidation with iodine in the presence of alcohols to phosphorothioate triesters, P (Z,V): and by oxidation with aqueous iodine to phosphorothioates, P (Z,V). Compound Xa can also be reacted with a mercaptan in the presence of an activator such as tetrazole to yield the dinucleoside phosphorothioite, VIIa, which can be chemically converted to XIIa by oxidation with sulfur to dinucleoside phosphorodithioates, P (Z,Y); and by oxidation with t-butylperoxide or aqueous iodine to phosphorothioates, P (L,Y). Thus it can be seen that the compounds XII and XIIa, as synthesized by process C, can be derived either from two intermediates, XIa and VIIa, or from one of these two intermediates. For example XIIa, where P (Z,Y) can be derived from either intermediate XIa or VIIa. For

XIIa where P (Z and X or W), XIa can be used to synthesize this class of compounds.

The present new compounds of structure II having different heteroatom containing substituents covalently linked to phosphorus can thus be prepared by processes A, B,and C. In some cases where Z and Y are linked to phosphorus and, therefore, yield a dinucleoside phosphorodithioate, processes A, B and C can all be used to prepare the same compound. This is also the case for certain other compounds such as II where Y and L are linked to phosphorus. Alternatively, compound II having Z and X or W or V (where V is covalently linked to phosphorus and to some group other than hydrogen as defined previously) linked to phosphorus can be synthesized by process C. Thus it can be seen that these processes lead to the synthesis of all the compounds described by II.

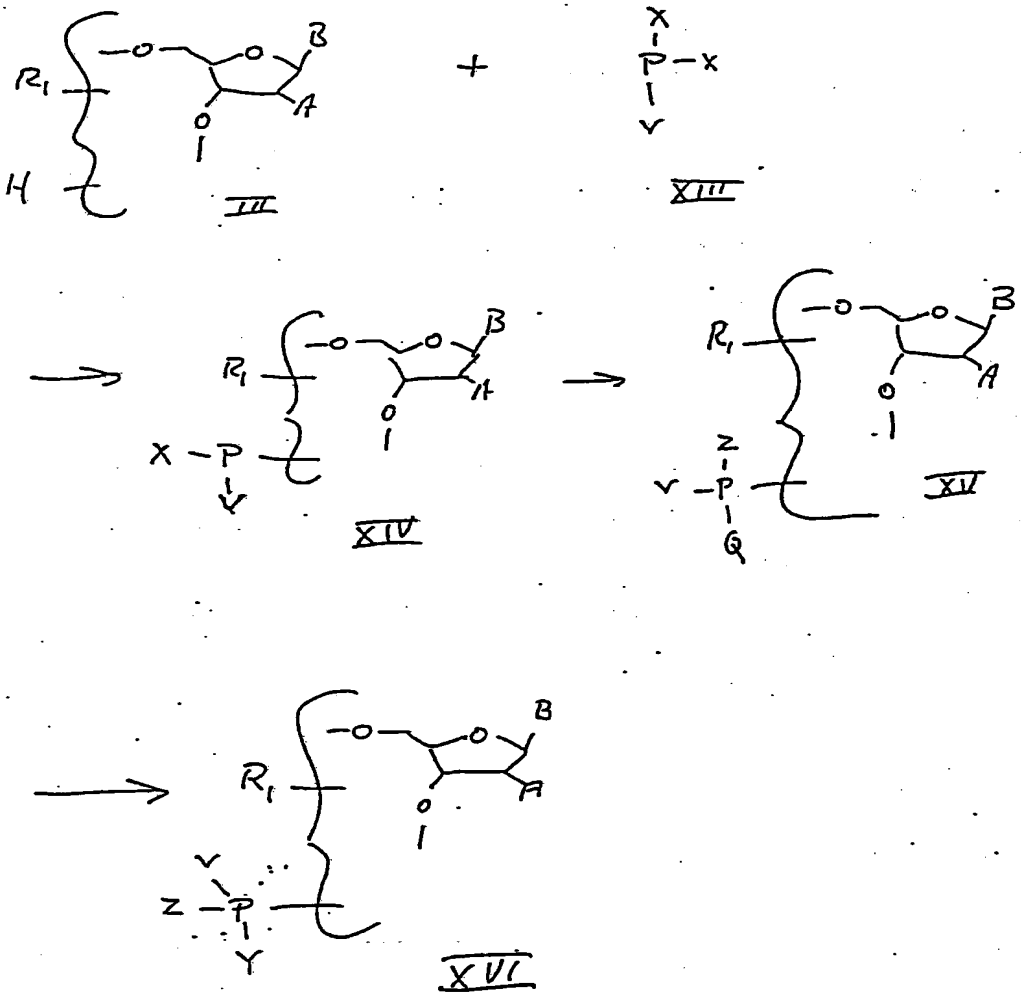
Compounds I, as the subset defined by V and Va, and II can then be used to synthesize polynucleotides having phosphorodithioate, phosphorothioamidate and phosphorothioate internucleotide linkages. These processes can be completed either on art form polymer supports or in the absence of these supports.

Of course the nucleoside moiety of the present invention can include more than one nucleoside and may include a number of nucleosides condensed as oligonucleotides having one or more phosphorus moieties (as shown in II) in combination with additional internucleotide phosphate diester linkages. These oligonucleotides may also only contain phosphorus noieties as shown in II. Polynucleotides having a mixture of internucleotide linkages including the presently described linkages as in II or only linkages as described in II are prepared using the new processes comprising one aspect of the present invention in combination with preferably conventional phosphoramidite methodologies

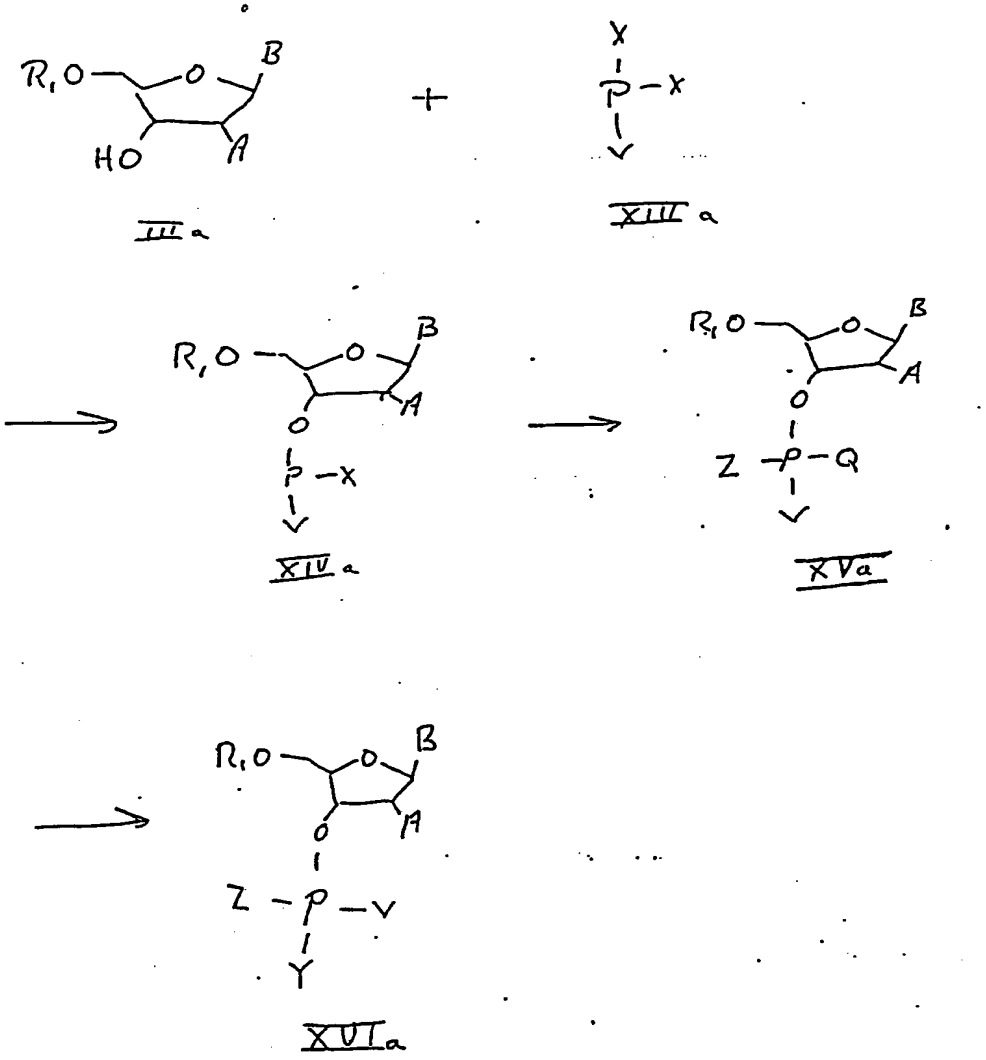
for synthesizing the other polynucleotide linkages (although other methods such as phosphate triester and phosphate diester and H-phosphonate procedures can also be used to synthesize these additional linkages). These condensation steps are best carried out on polymer supports although nonpolymer support procedures can also be used.

The present invention is particularly useful in the chemical synthesis of any deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) containing any deoxynucleotide, nucleotide, polynucleotide, or polydeoxynucleotide. Hybrid structures containing elements of deoxynucleotides and nucleotides in any combination as part of the same polynucleotide are also possible using compounds I and II. These new DNA or RNA compounds have analog substituents L, W. V, X, Y or Z covalently bonded to phosphorus at one or more internucleotide phosphorus containing linkages as found in DNA and RNA.

The synthesis of compounds according to general formula Ib can be represented by the following general reaction scheme, scheme D:


III
XIII
XIV
XV
XVI

The preferred reaction Scheme D is represented as follows:



wherein R_1, B, A, Q, X, Z, Y, W, and V are as previously described. Compounds XVI and XVIa are those in which all compounds have phosphorus double bonded to Z and also single bonded to V plus Y.

The process of scheme D involves synthesis of XIV and XIVa from IIIa and XIII or XIIIa. Reaction of XIV or XIVa with H_2S and an activator such as tetrazole yields a new compound, XVa, the nucleoside H-phosphonothioate, which can be chemically converted by oxidation with sulfur to nucleoside phosphorodithioates, P (Z, V, Y) and by alkylation of the nucleoside phosphorodithioate to the nucleoside phosphorodithioate triesters, P (Z, V, Y).

The preferred novel compounds according to the present invention are those compounds of general formula Ia and II wherein (for Ia) Y is a substituent having sulfur single bonded to phosphorus and to R_5 where R_5 is a heteroatom substituted or unsubstituted blocking group; A is H; R_1 is a blocking group, B is a nucleoside or deoxynucleoside base having art form blocking groups; and X is a secondary amino group; and (for II) Z is sulfur double bonded to phosphorus; Y is a substituent having sulfur single bonded to phosphorus and to R_5 where R_5 is a heteroatom substituted or unsubstituted blocking group; A is H; R_1 is a blocking group; B is a nucleoside or deoxynucleoside base having art-recognized blocking groups; and R_3 is H. These new compounds can then be used to prepare oligonucleotides having phosphorodithioate internucleotide linkages with P(Z,Y). These oligonucleotides are also preferred and novel new chemical entities.

The new compound II of the present invention can be prepared as shown in Scheme C from art-recognized starting materials such as IXa, a nucleoside 3'-phosphorodiamidite. The initial reaction is accomplished by dissolving the nucleoside in an organic solvent such as dioxane or tetrahydrofuran containing triethylamine to take up the liberated hydrochloric acid and adding a bis(dialkylamino)chlorophosphine. The resulting

nucleoside phosphorodiamidite is reacted without isolation with a second nucleoside. The isolated product of this reaction is a dinucleoside dialkylamino phosphoramidite, Xa, which can be reacted by two different pathways to form XIIa. The preferred pathway is to react Xa with a mercaptan in the presence of tetrazole to yield VIIa which is further treated with elementary sulfur to form the deoxydinucleotide phosphorodithioate, XIIa, where P(Z,Y). A second pathway is to treat Xa with hydrogen sulfide and tetrazole in an organic solvent such as acetonitrile to yield the dinucleoside H-phosphonothioate, XIa. Further reaction of the isolated dinucleoside H-phosphonothioate with elementary sulfur in an organic solvent such as a mixture of toluene and lutidine yields the dinucleoside phosphorodithioate, XIIa where P(Z,Y). Reaction of the dinucleoside phosphorodithioate with an alkyl or aryl halide capable of alkylating thiols yields the sulfur protected dinucleoside phosphorodithioate triester, XIIa. These new compounds of the present invention can then be used to synthesize polynucleotides having phosphorodithioate moieties at selected phosphorus internucleotide linkages. This is possible by first removing R_3 by conventional methods from XIIa to yield II and then reacting this compound with preferably an art-recognized phosphorodiamidite which leads to the dinucleotide 3'-phosphoramidite for use as a synthon in preparing polynucleotides. Compound II can also be converted to a dinucleotide 3'-phosphate, 3'-phosphate diester, or 3'-H-phosphonate. Synthesis of the polynucleotide can then proceed using any of these dinucleotide synthons on silica-based polymer supports using recognized procedures or in reaction solutions free of polymer supports.

As a further embodiment of the invention, the dinucleoside phosphorodithioates are preferably prepared by either reaction schemes A or B with A being preferred over B. These two reaction schemes differ in the method of preparing V and Va, the nucleoside phosphorothioamidite. For reaction scheme A, a bis (secondaryamino) chlorophosphine, which is prepared by standard procedures, is reacted with an appropriately protected nucleoside dissolved in acetonitrile and triethylamine. The resulting nucleoside diamidite, IXa, is then reacted without isolation with a mercaptan to yield the nucleoside thioamidite, Va, which is isolated by aqueous extraction and precipitation. For reaction scheme B, the mercaptyl-bis(dialkylamino)phosphine, IVa, is first formed and then condensed with the selected nucleoside in acetonitrile using tetrazole as an activator in order to form a nucleoside thioamidite, Va. Compound Va can then be condensed with a second nucleoside using an activator in order to form an S-aralkyldinucleoside phosphite, VIIa, which, after oxidation with elementary sulfur, yields VIIIa with P(Z, Y), the dinucleoside phosphorodithioate triester. These procedures shown in schemes A and B eliminate the requirement for dinucleoside phosphorodithioate triesters, as shown in scheme C, as synthons for preparing polynucleotides and are, therefore, preferred. Thus the nucleoside S-aralkyldialkylaminophosphoramidite or thioamidite (Va) and art-recognized nucleoside phosphoramidites can be used in any desired sequence in concert with either elementary sulfur or aqueous iodine oxidation procedures, respectively, to yield polynucleotides having any selected combination of phosphorodithioate and phosphate internucleotide linkages. By using only the S-aralkyldialkylaminophosphoramidite or thioamidite Va in concert with sulfur oxidation,

polynucleotides having only phosphorodithioate linkages can be prepared.

The synthesis of aralkylmercaptyl-bis-(dialkylamino)phosphine, IVb, is effected in an organic solvent solution whereby the bis(dialkylamino)-chlorophosphine, IVa, is first synthesized and then further condensed with an aralkylmercaptan. The first step is reacting phosphorus trichloride in an organic solvent such as tetrahydrofuran or dioxane with a five-fold excess of the dialkylamine. The reaction proceeds smoothly at reflux in a dry atmosphere of nitrogen or argon. The solution of the product is separated from the precipitated hydrochloride salt of the added amine, and can be concentrated under reduced pressure to a solid, if the dialkylamine is at least as large as diisopropylamine. This solid can then be recrystallized from chemically inert solvents such as pentane, hexane and heptane. Distillation of the bis(dialkylamino)chlorophosphine is also possible, especially for lower molecular weight compounds. These bis secondaryamino chlorophosphines can then be used directly to form compound IXa (schemes A and C) or for synthesizing IVb. For the synthesis of IVb, the next step involves dissolving an aralkylmercaptan in an inert solvent such as ethyl ether, tetrahydrofuran or dioxane; adding an equivalent of sodium hydride in order to convert the mercaptan to the mercaptide; and finally adding the bis(dialkylamino) chlorophosphine to the reaction mixture. The S-aralkylmercaptyl-bis(dialkylamino)-phosphine is formed quantitatively over several hours at room temperature. Removal of sodium chloride followed by crystallization from solvents such as acetonitrile affords the desired product. If the product, IVb, cannot be crystallized then purification may be possible by vacuum distillation.

However; if distillation leads to decomposition, then the nucleoside thioamidite should be synthesized by the preferred method using scheme A which does not require the synthesis of IVb as an intermediate.

Synthesis of internucleotide bonds containing phosphorodithioate linkages where IVb is used for this conversion requires activating agents which are proton donors. Thus, these phosphines are activated by acidic compounds through protonation which facilitates the formation of the desired internucleotide bonds containing initially a thiophosphite triester. The initial activation step involving IVb requires acidic species, preferably mildly acidic, and include tetrazole and 3-nitrotriazole. The resulting nucleoside thioamidite, Va, may be difficult to activate and require more acidic species such as aromatic amine salts of strong acids, para-nitrophenyltetrazole, pyridinium tetrafluoroborate, trifluoromethylphenyltetrazole and trifluoromethyltetrazolide salts. This is especially the case where X is diisopropylamino. However, when the nucleoside thioamidite contains a less sterically hindered X such as dimethylamino or pyrrolidino, then activation with a much milder acid such as tetrazole is possible and is preferred. These less sterically hindered nucleoside thioamidites are most easily prepared via reaction scheme A.

The mercaptyl moiety can vary considerably in structure. The criteria are that it facilitate activation of Va and that it is easily removed after completion of the synthesis of a polynucleotide. Thus, the preferred mercaptans include benzyl and heteroatom substituted benzyl moieties such as 2,4-dichlorobenzyl, phenyl and heteroatom substituted phenyl, and heteroatom substituted or unsubstituted alkyl substituents such as
β-cyanoethyl and methyl.

The secondary amino moieties as part of the phosphines IVa and IVb and the nucleoside thioamidites, Va, are preferably substitutents that stabilize these intermediates toward storage and synthesis. These secondary amino groups should also preferably facilitate activation of the phosphine during the reactions leading to the formation of internucleotide bonds. These criteria are met most easily by substituents such as dimethylamino, diethylamino, diisopropylamino, dipropylamino, dibutylamino, dipentylamino, pyrrolidino, piperidino, various isomeric alkyl groups, and also aralkyl groups.

When the present new compounds are used to form polynucleotides, they are employed in combination with art recognized nucleoside phosphoramidites or in the absence of nucleoside phosphoramidites. Thus at sites where normal phosphate diester linkages are to be inserted into polynucleotides, art recognized procedures such as activation with tetrazole, oxidation with aqueous iodine, capping with acetic anhydride if synthesis is on art recognized polymer supports, and detritylation with acid are used for synthesis. At the sites where phosphorodithioate linkages are to be incorporated into polynucleotides, a nucleoside thioamidite, Va, is activated with tetrazole, aromatic amine salts, pyridinium tetrafluoroborate, para-nitrophenyl tetrazole, trifluoromethylaryl tetrazole or similar reagents, and following coupling to the growing polynucleotide, the thiophosphite internucleotide linkage is oxidized, preferably with elementary sulfur to yield the dithioate. Other steps for utilizing Va in the polynucleotide synthesis are the same as with art recognized nucleoside phosphoramidites. When DNA containing only phosphorodithioate linkages is to be prepared, Va is activated, condensed, and oxidized

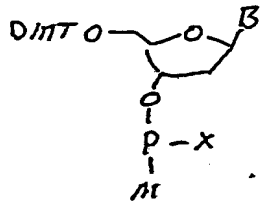
with sulfur as described above, repetitively with a nucleoside preferably attached to a polymer support to yield polynucleotides having phosphorodithioate linkages. Dinucleoside phosphorodithioate triesters VIIIa or XIIa where P(Z,Y) can also be used as synthons for polynucleotide synthesis. These new compounds are prepared using the presently described new processes. After conversion to preferably protected dinucleoside phosphorodithioate 3'-phosphoramidites, they can be activated with tetrazole and used directly as dinucleotide synthons via normal art recognized polynucleotide synthesis procedures, either preferably on polymer supports or in the solution phase in the absence of polymer supports.

Of course once the internucleotide bonds of the polynucleotide have been synthesized, which includes both normal linkages and the phosphorodithioate linkages, or polynucleotides having exclusively phosphorodithioate linkages, the product can, if desirable, be freed of blocking groups. Thus the first step is treatment with preferably trialkylammonium thiophenolate to remove the aralkyl blocking group from the dithioate moiety or, if methyl groups are used to protect either normal or phosphorodithioate internucleotide linkages, the methyl group from these triesters. The remaining blocking groups on sugars, bases or phosphorus, and also the linkage joining the polynucleotide to a support if the synthesis had been completed in this manner, can then be removed using art recognized procedures such as hydrolysis with aqueous ammonia. If blocking groups on sulfur are used that are labile to reagents other than thiophenolate (i.e., trichloroethyl or β-cyanoethyl), then the deprotection protocol should be modified accordingly.

The following examples and procedures depicting the formation of the compounds according to the present invention are presented in order to provide a more complete understanding and illustration of the present invention.

EXAMPLE I

Bis(dimethylamino)chlorophosphine was prepared by adding tris(dimethylamino)-phosphine (36.3 ml, 32.6 g, 0.2 mole) and trichlorophosphine (8.7 ml, 13.7 g, 0.1 mole) to anhydrous ether (100 mol). After stirring for 3 hours at room temperature, solvent was removed by concentration in vacuo at room temperature. The product was then distilled (b.p. 72-75°C) at reduced pressure (approx. 16 mm Hg) using a water aspirator to yield 30 g. of product. ^{31}P-NMR($CHCl_3$) δ 163.06. This procedure is also used to produce dipyrrolidinylchlorophosphine. Preparation of thiophosphoramidites of the formula



represented as Va (Reaction Scheme A) where

B = 1-Thyminyl;

B = 1-(N-4-benzoylcytosinyl;

B = 9-(N-6-benzoyladeninyl);

B = 9-(N-2-isobutyrylguaninyl); and

DMT = di-p-anisylphenylmethyl (dimethoxytrityl)

M = 4-chlorobenzyl or 2,4-chlorobenzyl

X = N,N-dimethylamino or pyrrolidinyl

and the further use of these compounds to prepare oligonucleotides having phosphorodithioate internucleotide linkages.

The following example describes the synthesis of 5'-O-dimethoxytrityl-N4-benzoyldeoxycytidylyl-3'-S(4--chlorobenzyl) phosphorthiopyrrolidinite. The same procedure can be used for the other suitably protected deoxynucleosides. Similarly the same procedure is

useful for the 2.4-dichlorobenzyl and 4-chlorobenzyl protected sulfur derivatives and for the N,N-dimethylamino and pyrrolidinyl amidites. Table 1 summarized the ^{31}P-NMR data for all these amidites.

5'-O-Dimethoxytrityl-N4-benzoyldeoxycytidine (317 mg, 0.5 mmol) was dissolved in acetonitrile (2 ml) and triethylamine (1 ml) under argon. Bispyrrolidinylchlorophosphine (124 mg, 0.6 mmol) was added which was followed by the immediate formation of a precipitate (^{31}P-NMR of the reaction product was at 133.8 ppm). After 5 minutes stirring at room temperature, 4-chlorobenzylmercaptan (159 mg, 1mmol) was added to the reaction mixture and the solution, including the precipitate, was concentrated to a glass in vacuo at room temperature. The glass was resuspended in acetonitrile (2 ml). The ^{31}P-NMR spectrum of the reaction mixture indicated that the major phosphorus containing product was the diastereoisomers of the thioamidite (161.5, 159.7 ppm). Minor impurities were an adduct of bispyrrolidinylchlorophosphine and 4-chlorobenzylmercaptan (107.0 ppm) and hydrolysis products (12.4 ppm). Triethylamine was next added to the reaction mixture. The solution was diluted with deacidified ethylacetate (50 ml) and extracted with aqueous saturated sodium bicarbonate (50 ml x 2) and brine. The combined aqueous solutions were back-extracted with deacidified ethylacetate (10 ml). The organic solutions were combined, dried for 1 hour over sodium sulfate in the presence of 10% (volume) triethylamine, filtered, and the filtercake washed with 5 ml deacidified ethylacetate. The organic solution was then concentrated in vacuo to a white foam. This foam was dissolved in toluene (10 ml) containing 1% triethylamine and the product isolated by precipitation into n-pentane: triethylamine (999:1, v/v). After filtration, the product was

dried in vacuo over phosphorus pentoxide and potassium hydroxide and isolated in 83.1% yield (741 mg).

^{1}H-NMR ($CDCl_3$) 8.76 (broad s, 1H, NH), 8.37 (d, JHH = 7.47 Hz, 0.5H, H5, cytosine), 8.31 (d, JHH = 7.48 Hz, 0.5H, H5, cytosine), 7.94 (d, JHH = 7.37 Hz, 2H, H2 and H6 of benzoyl group), 7.68-7.54 (m, 3H, H3, H4, H5 of benzoyl group), 7.44-7.14 (m, 14H, aromatic protons of 4-chlorobenzyl group, H2, H6 of anisyl (DMTr), ph-protons (DMTr), H6 cytosine)), 6.91 (d, JHH = 7.57 Hz, 4H, H3, H5 of anisyl DMTr), 6.33 (m, 1H, 1'H), 4.72 (m, 1H, 3'H), 4.22 (m, 1H, 4'H), 3.84 (d of singletts, 6H, methyl protons of anisyl DMTr), 3.84-3.76 (m, 2H, methylene protons of 4-chlorobenzyl group), 3.59-3.35 (m, 2H, 5'H), 3.19-3.01 (m, 4H, methylene protons of pyrrolidinyl group a to nitrogen), 2.84-2.75, 2.37-2.26 (m, 2H, 2'H), 1.79-1.71 (m, 4H, methylene protons of pyrrolidinyl group b to nitrogen). ^{31}P-NMR ($CDCl_3$) 161.79, 159.97. Fab$^+$: 923 $(M + S)^+$, 907 $(M + O)^+$. tlc: R_f .75 (chloroform:ethylacetae:triethylamine (45:45:10, v/v/v).

Using a deoxynucleoside attached covalently to a silica based polymer support through the 3'-hydroxyl (U.S. Patent 4,458,066), synthesis of deoxyoligonucleotides containing phosphorodithioate linkages proceeded according to the reaction sequence outlined in Figure 1. Synthesis began by reacting a dry acetonitrile solution of any compound Va (10 equivalents) and tetrazole (50 equivalents) with 1 μ mole of deoxynucleoside on silica for 30 sec (step i) followed by a 400 sec oxidation with 5% sulfur in pyridine:carbon disulfide (1:1, v/v, step ii). Coupling was performed twice to ensure high yields (greater than 98%. Acylation of unreactive deoxynucleoside (step iii), detritylation (step iv) and various washes were the same as those described

previously for synthesizing natural DNA from deoxynucleoside phosphoramidites (U.S. Patent 4,415,732 and Science 230, 281-285, 1985). Multiple repetitions of this cycle then led to the synthesis of DNA containing exclusively phosphorodithioate linkages or, when used in combination with deoxynucleoside phosphoramidities, to deoxyoligonucleotides having both phosphorodithioate and phosphate internucleotide bonds.

Synthetic deoxyoligonucleotides were isolated free of protecting groups via a two-step protocol (thiophenol:triethylamine:dioxane, 1:1:2, v/v/v for 24 h followed by conc. ammonium hydroxide for 15h) and then purified to homogeneity by standard procedures (polyacrylamide gel electrophoresis and reverse phase hplc). ^{31}P-NMR spectra (Figure 2) of phosphorodithioate DNA indicated that this synthesis protocol yielded DNA containing exclusively phosphorodithioate internucleotide linkages. No hydrolysis of these dithioates to phosphorothioates (^{31}P-NMR 56) or phosphate was observed. So far a pentadecamer homopolymer containing fourteen dithioate linkages, lac and cro operators (O_r1) with multiple dithioates at defined sites, and a cro operator segment (O_r1) containing seventeen contiguous dithioates have been synthesized.

Figure 1. Synthesis of DNA on a Polymer Support. Ⓟ , silica based polymer support.

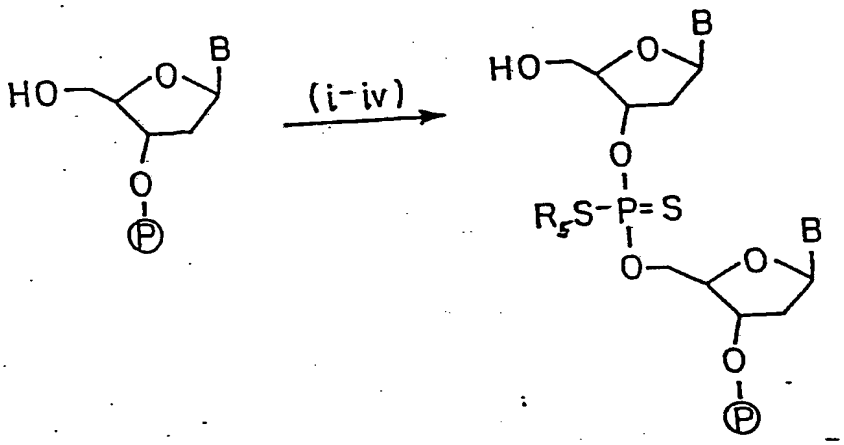
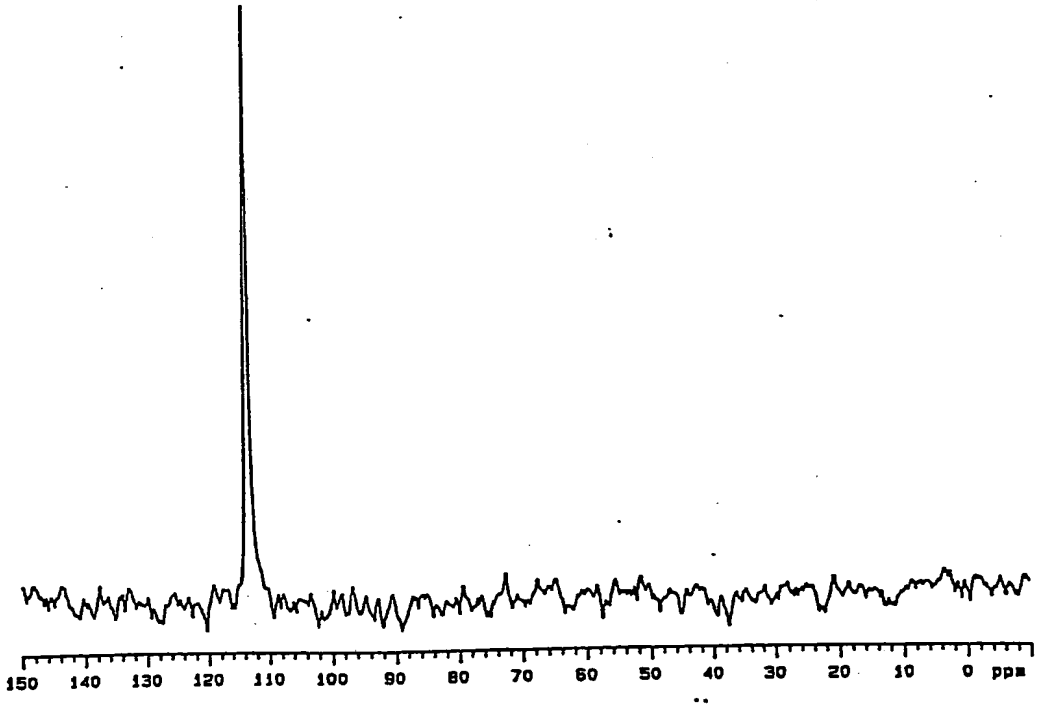
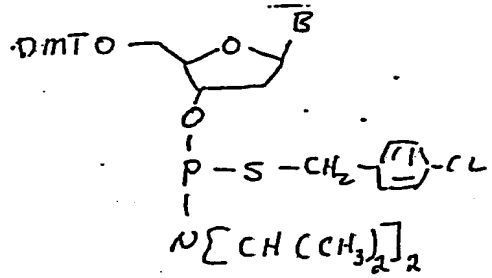


Figure 2. ^{31}P-NMR Spectra of a Polynucleotide Derivatives. Spectra of $d(C)_{15}$ containing exclusively phosphorodithioate internucleotide linkages (113 ppm in D_2O). ^{31}P-NMR spectra was recorded on a Varian VXR-500S. Aqueous 85% H_3PO_4 was the external standard.



EXAMPLE II

Preparation of thiophosphoramidites of the formula:



represented as Va (Reaction Scheme B)

B = 1-Thyminyl;

B = 1-(N-4-benzoylcytosinyl);

B = 9-(N-6-benzoyladeninyl);

B = 9-(N-2-isobutyrylguaninyl); and

DMT = dimethoxytrityl

The synthesis of compounds Va begins with the preparation of 4-chlorobenzylmercaptyl-bis-(diisopropylamino)phosphine. Phosphorus trichloride (0.5 mole, 68,665 g, 43.6 ml) was dissolved in 300 ml anhydrous tetrahydrofuran (THF). The PCl_3 solution was cooled to -18°C by a NaCl ice mixture. Diisopropylamine (2.5 mole, 252.983 g, 350.4 ml) was then added slowly via a dropping funnel. At first the reaction was violent and had to be carried out under vigorous stirring (mechanical stirrer) and cooling. After the reaction to the bis-(diisopropylamino) chlorophosphine was complete, the reaction mixture was refluxed for 12 hours to afford the desired product. After 12 hours the reaction mixture was cooled to rt and the diisopropylammonium chloride was removed by filtration through a Schlenk-fritt. After washing the salts with THF, the clear reaction mixture was refluxed again for 12 hours to afford the desired product as the only phosphorus containing material in the reaction mixture (^{31}P-NMR 132.4 ppm). The newly formed diisopropylammonium chloride

was removed by filtration and washed with anhydrous ether. The filtrate was evaporated under reduced pressure (rotary evaporator) to a yellowish solid which was recrystallized from hexanes to afford a colorless crystalline solid. This compound was air stable and moisture sensitive. 4-Chlorobenzyl-mercaptan (50 mmol, 7.93 g, 6.6 ml) was dissolved in anhydrous ether (300 ml) and an amount of a sodium hydride suspension in oil (50% NaH in oil) equivalent to 50 mmol (2.4 g) was added to the mercaptan solution. As the solution was stirred (magnetic stirrer), hydrogen evolved indicating the formation of sodium 4-chlorobenzylmercaptide. After two hours, bis-(diisopropylamino)chlorophosphine (50 mmol, 13.34 g) was added and the reaction mixture was stirred until gas evolution stopped (4 hours at rt). ^{31}P-NMR of the reaction mixture indicated quantitative conversion of the chlorophosphine to the desired product without any side reactions (^{31}P-NMR 91.4ppm). The salt (sodium chloride) was removed by filtration through a Schlenk fritt and washed with anhydrous ether (50 ml). The colorless filtrate was evaporated to a white foam (4-chlorobenzylmercaptyl-bis-(diisopropylamino)phosphine) which was dissolved in a minimum amount of hot acetonitrile (100 ml) and recrystallized from the same solvent to afford a white crystalline product.

The 5'-O-dimethoxytrityl nucleoside (5 mmol) and 4-chlorobenzylmercaptyl-bis-(diisopropylamino)phosphine (6 mmol, 2.33g) were suspended in dry acetonitrile (15 ml). Tetrazole (10 mmol, 0.69 g) was added and the reaction was stirred for 16 hours at room temperature. The initially present solids (phosphine and nucleoside) dissolved during the reaction time and a crystalline solid (diisopropylammonium tetrazolide) precipitates. After 16 hours, the reaction was quenched with pyridine (1 ml) and diluted into acid

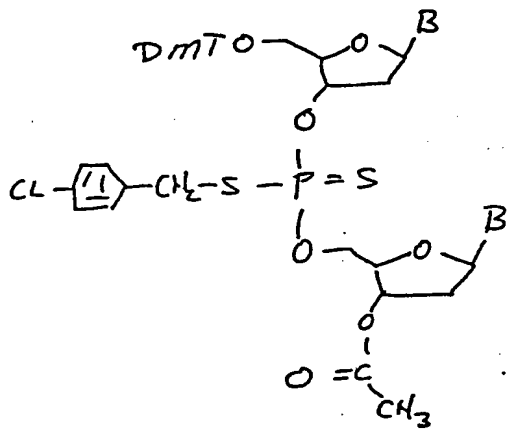
free ethylacetate (100 ml). The solution was extracted twice with an aqu ous saturated solution of sodium bicarbonate and once with brine, successively.

The organic layer was dried over sodium sulfate. After removal of this salt, the solvent was evaporated in vacuo to afford a glass which was redissolved in a mixture of chloroform, ethylacetate and triethylamine (45:45:10, v/v/v) and chromatographed on silica gel with the same solvent. Column chromatography fractions containing the desired product were combined and the solvent evaporated in vacuo. The product was dissolved in toluene and precipitated into n-pentane. The nucleoside phosphorthioamidite was isolated after drying the precipitate in vacuo over P_2O_5/KOH (3.33 g, 80.1% yield).

^{31}P NMR 161.3 and 159,97 ppm (two diastereomers) with respect to external standard of H_3PO_4 for the thymidine derivative. ^{1}H NMR 8.0 (N-H), 7.59 and 7.58 (2 x d, J_{HH} = 1.2 Hz), 7.42-7.19 (m), 6.83 (d, J_{HH} = 8.7 Hz), 6.37 (q, H_1'), 4.65-4.58 (m, $H_{3'}$), 2.05-1.83 (m, $H_{6'}$), 3.80-3.61 (m, CH_2 of p-chloro benzyl), 3.78 (s, H_6), 3.48-3.29 (m. $H_{5'}$), 2.45-2.24 (m, H_2'), 1.44 (CH_3-T), 1.17-1.04 (m, CH_3 of isopropyl).

EXAMPLE III

Synthesis of Dinucleoside Phosphorodithioate Triesters of the formula:



represented as VIIIa (Reaction Scheme B) where

B = 1-Thyminyl;

B = 1-(N-4-benzoylcytosinyl);

B = 9-(N-6-benzoyladeninyl);

B = 9-(N-2-isobutyrylguaninyl); and

DMT = dimethoxytrityl

5'-O-dimethoxytritylthymidine-3'-S-(4-chlorobenzyl)diisopropylaminophosphoramidite (compound Va, example II) (0.2 mmol, 166.3 mg) and 3'-O-acetylthymidine (0.2 mmol, 56.8 mg) were dissolved in anhydrous dimethylformamide (2 ml). 4-Nitrophenyltetrazole (1 mmol, 191.2 mg) was next added to this solution. After 15 minutes the reaction to the dinucleoside thiophosphite was quenched with sulfur (1 atomic equivalent, 32 mg). The reaction mixture was then diluted with ethylacetate (50 ml) and the sulfur removed by filtration through a cotton plug. After removal of the solvents in high vacuo, the desired product was dissolved in

ethylacetate (10 ml) and extracted twice with an aqueous saturated solution of sodium bicarbonate and once with brine successively. The organic layer was dried over sodium sulfate. After removal of the salt, the product was chromatographed on silica with a mixture of 1.1.1-trichloroethane and methanol (92.5:7.5, v/v). The product fractions were combined and the solvent removed in vacuo. The dinucleoside phosphorodithioate was dissolved in toluene and precipitated into n-pentane (^{31}P NMR 97.8, 96.2 with respect to 85% H_3PO_4 as an external standard). FAB^- mass spectrum, 1047 (M^-), 921 (-p-chlorobenzyl), 743 (-DMT), 619 (-DMT and 4-chlorobenzyl), 519 (3'-O-acetylthymidine 5'-O-4-chlorobenzyl phosphorodithioate), 395 (3'-O- acetylthymidine 5'-O-phosphorodithioate).

The 4-chlorobenzyl group was removed from the phosphorodithioate triester with a mixture of dioxane:triethylamine:thiophenol (2:2:1, v/v/v) within 1.5 hours at room temperature.

These dinucleoside phosphorodithioate triesters can also be prepared by using pyridinium tetrafluoroborate as an activating agent. Pyridinium tetrafluoroborate was prepared by dissolving HBF_4 (10 mmole, 1.9 g of a diethyletherate, Aldrich Chemical Co.) in dry dichloromethane (5 ml) and adding this solution with stirring to dry pyridine (791 mg, 10 mmole) in dry ethyl ether (50 ml). After 2 h the salt was removed by filtration, washed with dry ethyl ether, and dried in a dessicator over P_2O_5. In a typical reaction, 3'-O-acetylthymidine (142 mg. 0.5 mmole) was allowed to react with 5'-O-dimethoxytritylthymidine-3'-S(4-chlorobenzyl) diisopropylaminophosphoramidite (833 mg. 1 mmole) in the presence of pyridinium tetrafluoroborate (334 mg, 2 mmole) in dry acetonitrile (5 ml). After ten minutes the reaction mixture was quenched by addition of 20

atomic equivalents of sulfur (640 mg) in pyridine (2 ml), concentrated in vacuo to a gum, redissolved in ethylacetate (50 ml), and the excess sulfur removed by filtration. Following the standard aqueous work-up, as described previously in this example, and column chromatography (CH_3CCl_3: CH_3OH, 95:5, v/v), the dinucleoside phorphorodithioate in protected form was isolated by precipitation into pentane (60% yield). The following dinucleoside phosphorodithioates in approximately 60% yield have been prepared via this procedure.

a.) 5'-O-Dimethoxytritylthymidine S-(4-chlorobenzyl)-3'-O-(5'-O-thymidylyl)-phosphorodithioate. FAB^+ mass spectrum, 1005 (M^+), 847 (M - 4-chlorobenzylmercaptyl), 703 $(M - DMT + H)^+$, 455 $(M - DMT\text{-}4\text{-}chlorobenzylmercaptyl + H)^+$; FAB^- mass spectrum, 879 $(M - 4\text{-}chlorobenzyl)^-$, 779 (M - 5'-thymidylyl), 477 (thymidine-S-4-chlorobenzyl-phosphorodithioate), 355 (thymidine 5'-phorphorodithioate); ^{31}P NMR ($CDCl_3$) 96.44 UV (EtOH) max 228, 268 nm.

b.) 5'-O-Dimethoxytritylthymidine S-(4-chlorobenzyl)-3'-O-(5'-O-N2-isobutyryldeoxyguanosinyl)- phosphorodithioate. FAB^+ mass spectrum, 1277 $(M - Na)^+$, 952 $(M - DMTr)^+$; ^{31}P-NMR ($CDCl_3$) 95.8, 96.14; UV (EtOH) max 262 nm.

c.) 5'-O-Dimethoxytrityl-N6-benzoyldeoxyadenosine S-(4-chlorobenzyl)-3'-O-(5'-O-N4-benzoyldeoxycytidine)-phosphorodithioate. ^{31}P NMR ($CDCl_3$) 93.89, 93.31.

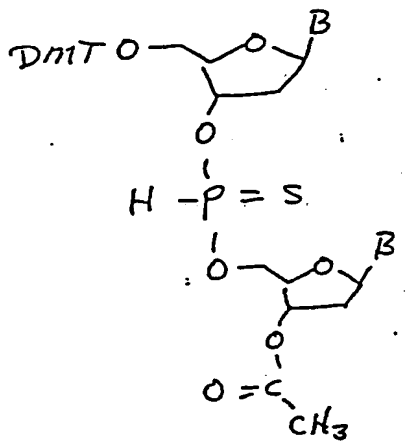
Synthesis of dinucleoside phosphorodithioates, especially with strong acid catalysts such as 4-nitrophenyltetrazole or pyridinium tetrafluoroborate, should be carried out under an inert atmosphere. Handling in air leads to the formation of various amounts of the corresponding oxides. Also compounds tentatively assigned as the

4-chlorobenzylphosphonothioamidates are formed when phosphorothioamidites are r acted with acidic catalysts. These reactions, however, do not necessarily interfere with coupling as complete conversion of the 3'-protected deoxynucleoside to the dinucleoside thiophosphite can be achieved by using an excess of the thioamidite and high concentrations of both deoxynucleoside derivatives. Preliminary investigations have also revealed that the resulting thiophosphite triesters are stable toward nonnucleophilic base and undergo rapid acid catalyzed hydrolysis to hydrogen phosphonates. They are susceptible to rapid oxidation by air or

t-butylhydroperoxide to yield phosphorothioates and by sulfur to the phosphorodithioate triester.

EXAMPLE IV

Synthesis of Dinucleoside H-Phosphonothioate of the formula:



represented as XIa (Reaction Scheme C) where

B = 1-Thyminyl;

B = 1-(N-4-benzoylcytosinyl);

B = 9-(N-6-benzoyladeninyl);

B = 9-(N-2-isobutyrylguaninyl); and

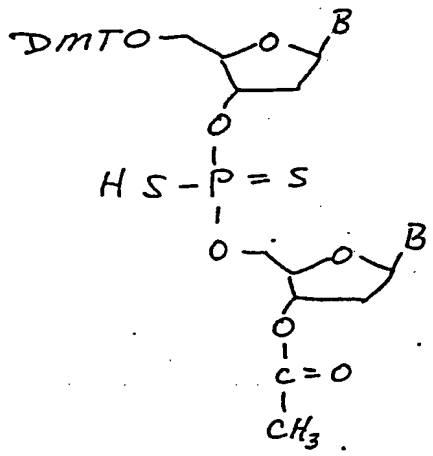
DMT = dimethoxytrityl

The first step was condensation of 5'-O-dimethoxytritylthymidine with bis(diisopropylamino)-chlorophosphine in dioxane containing triethylamine. The resulting phosphorodiamidite was reacted without isolation with 3'-O-acetylthymidine to yield a homogeneous dinucleoside amidite in 62% yield after silica gel chromatography (5% triethylamine in ethylacetate). Synthesis of the dinucleoside H-phosphonothioate proceeds by dissolving the dinucleoside phosphoroamidite (470 mg, 0.5 mmol) in acetonitrile (5 ml), bubbling H_2S through the solution for 1 min, adding tetrazole (35 mg, 0.5 mmol in 1 ml acetonitrile), and finally stirring the sealed reaction flask for 16 h. The reaction mixture was

concentrated to a gum on a rotary evaporator, redissolved in ethylacetate (50 ml) and extracted twice with 2 M triethylammonium bicarbonate (pH 7.4, 20 ml each). After concentrating in vacuo to a gum, the product was dissolved in dichloromethane (5 ml) and isolated by precipitation into pentane (400 mg, 90%). FAB^+ mass spectrum, 527 (anhydro DMT dT): FAB^- mass spectrum, 890 (M^-), 623 (DMT dT-3'-PHO_2^-), 363 (M-527, 5'-PHO_2^--dT-3'-OAc); 31 NMR 71.7 and 70.7 ($^1J_{HP}$ = 673.8 Hz and 676.3 Hz; ^1H NMR 7.81 and 7.80 (P-H, $^1J_{HP}$ = 671.4 Hz and 676.7 Hz), 7.55 and 7.53 (s, H_6), 7.37-7.20 (m, aromatic), 6.82 (d, J = 8.8 Hz, DMT), 6.49 and 6.26 (m, $H_{1'}$), 5.49 and 5.25 (m, $H_{3'}$), 4.35 (m, $H_{4'}$), 4.19 (m, $H_{5'}$), 4.07 (m, $H_{4'}$), 3.76 (s, MeO-DMT), 3.42 (m, $H_{5'}$), 2.54-2.32 (m, $H_{2'}$), 2.08 and 2.07 (2 x s, CH_3-acetyl), 1.90 (m, CH_3-T), 1.43 (s, CH_3-T). R_f = 0.35 and 0.28 (methanol/-dichloromethane, 1:9, v/v).

EXAMPLE V

Synthesis of a Dinucleoside Phosphorodithioate of the formula:



represented as XIIa,P(Z,Y), (Reaction Scheme C) where

B = 1-Thyminyl;
B = 1-(N-4-benzoylcytosinyl);
B = 9-N-6-benzoyladeninyl);
B = 9-(N-2-isobutyrylguaninyl); and
DMT = dimethoxytrityl

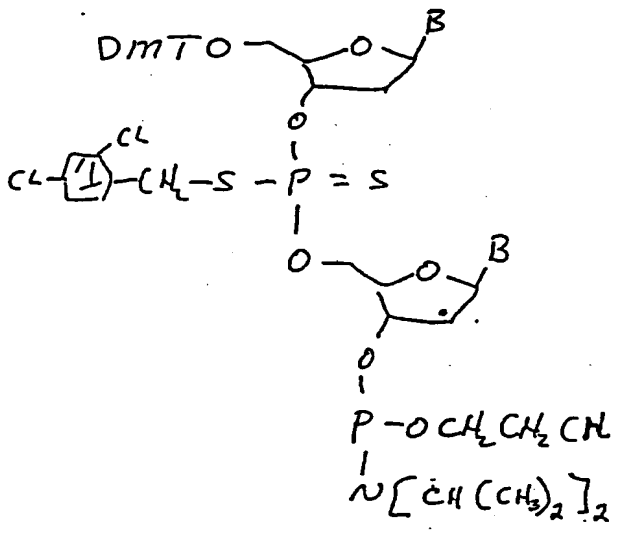
Dithymidine phosphorodithioate was synthesized by stirring the dinucleoside H-phosphonothioate (104 mg. 0.1 mmol in 1 ml dichloromethane) with elementary sulfur (1 mmol in 2 ml toluene:2,6-lutidine, 19:1, v/v) for 0.5 h. Purification via silica gel column chromatography (0-12% methanol in dichloromethane and 0.5% triethylamine) afforded 70% isolated yield. FAB^+ mass spectrum, 303 (DMT^+); FAB^- mass spectrum, 921 (M^-), 395 (5'-PS_2O^--dT-3'-OAc); ^{31}P NMR 112.7; ^1H NMR 8.12 (s, NH), 7.90 and 7.60 (2 x s, H_6), 7.40-724 (m, aromatic), 6.80 (d, J_{HP} = 8.8 Hz, DMT), 6.43 (m, H_1,), 5.46-5.36 (m, H_3'), 4.40 (m, H_4'), 4.16 (m, H_5,), 3.76 (s, MeO-DMT), 3.52 (m, H_5'), 2.28 (m, H_2,), 2.05 (Ch_3-acetyl), 1.97 (CH_3T), 1.58 (s,

CH_3T). R_f = 0.14 (methanol/dichloromethane, 1:9, v/v).

The dinucleoside phosphorodithioate was deprotected by standard procedures and isolated in 86% yield after ether extractions (3x), sephadex G10 gel filtration (H_2O), and lyophilization as the ammonium salt. FAB^+ mass spectrum, 579 (M); ^{31}P NMR (D_2O) 113.3; ^1H NMR 7.60 and 7.46 (2 x s, H_6), 6.11 and 5.99 (m, $H_{1'}$), 5.17 (m, H_3'), 4.85 (m, $H_{3'}$), 4.15 (m, H_4'), 4.03 and 3.62 (m, H_5'), 2.21 (m, H_2'), 1.88 m, CH_3-T). R_f = 0.25 (methanoltriethylamine/chloroform, 15:1:84, v/v/v). When the dinucleoside phosphorodithioate was phosphorylated with T4-polynucleotide kinase and [γ-^{32}P]ATP, the rate of kination was approximately one-half that of unmodified 3'-5' dithymidine phosphate under identical conditions. Further testing with snake venom phosphodiesterase (Crotalus adamanteus venon, Sigma) indicated that the phosphorodithioate was stable using conditions where the natural dinucleotide was completely hydrolyzed (assayed by reverse phase HPLC). This compound was also observed to be stable to conc. ammonium hydroxide at 55°C (16 h) as no degradation or isomerization was observed (^{31}P NMR, thin layer chromatography).

EXAMPLE VI

Synthesis of a Dinucleoside Phosphorodithioate 3'-Phosphoramidite of the formula:



represented as XVIIa where

B = 1-Thyminyl;

B = 1-(N-4-benzoylcytosinyl);

B = 9-(N-6-benzoyladeninyl);

B = 9-(N-2-isobutyrylguaninyl); and

DMT = dimethoxytrityl

In order to introduce the phosphorodithioate linkage into oligonucleotides, a protection/-deprotection scheme for the phosphorodithioate internucleotide linkage was developed. Thus the dinucleoside phosphorodithioate in protected form (XIIa) (57 mg, 0.06 mmol) was alkylated with α,2,4-trichlorotoluene (50 µl, 1 h, 55°C) in acetonitrile to yield the dinucleoside phosphorodithioate triester quantitatively. Further testing revealed that it was completely stable to reagents used in DNA synthesis (1% trifluoroacetic acid in dichloromethane and iodine in aqueous lutidine/THF) and that the phosphorodithioate triester was

specifically S-dealkylated by treatment with thiophenolate (thiophenol:triethylamine:dioxane, 1:1:2, v/v/v, $t_{\frac{1}{2}}$ = 3 min at rt). FAB^+ mass spectrum, 527 (anhydro DMT dT); FAB^- mass spectrum, 923 (M + 1-dichlorobenzyl), 813 (DMT dT-3'-PSOS-dcb), 553 (5'-PSOS-dcb-dT-3'OAc); ^{31}P NMR (CH_3CN, ext. lock) 94.4 and 93.7, ^1H NMR 7.55 and 7.52 (2 x s, H_6), 7.37-7.23 (m, aromatic), 681 (d, J = 4.6 Hz, DMT), 6.34 and 6.28 (m, $H_{1Y'}$), 5.38 and 5.01 (m, H_3'), 4.24-4.08 (m,CH_2-benzyl, H_5') + H_4'), 3.76 (s,MeO-DMT), 3.42 (m,H_5'), 2.39 (m,H_2'), 2.08 (s, CH_3-acetyl), 1.89 and 1.87 (2 x s CH_3-T), 1.43 and 1.42 (2 s, CH_3-T). R_f = 0.74 (methanol/-triethylamine/chloroform, 15:1:84, v/v/v).

Conversion to a synthon useful for DNA synthesis was a two step process. The dinucleoside phosphorodithioate triester was first deacylated (the 3' acetyl group) using 0.15 M tert-butylamine in methanol (0°C, 10 h) and purified by silica gel chromatography to yield IIa. Less than 5% cleavage of the internucleotide linkage (^{31}P NMR, TLC) was observed. The deacylated compound was then reacted with bis(diisopropylamino)-2-cyanoethoxy phosphine (1.5 eq) in the presence of tetrazole (1 eq, 1 h at rt) to yield the dinucleoside phosphorodithioate triester as the 3'-phorphoramidite in 76% yield. ^{31}P NMR 149.4, 149.2, 148.9 and 97.2, 95.7, 95.5. ^1H-NMR 7.56 (s, H_6), 7.33-7.27 (m, aromatic), 6.84 (d, J = 8.5 Hz, DMT), 6.39-6.29 (m, H_1'), 5.44 (m, H_3'), 3.79 (s, MeO-DMT), 1.90 (s, CH_3-T), 1.45 (s, CH_3-T),1.18 (d, J = 6.6 Hz, CH_3-iPr). R_f = 0.29 and 0.17 (chloroform/ethylacetate/triethylamine, 45:45:10, v/v/v). The resulting dinucleotide phosphoramidite, XVIIa, has been used successfully in combination with unmodified mononucleoside phosphoramidites for the synthesis of 26-mer DNA fragments containing the phosphorodithioate linkage (98.2%

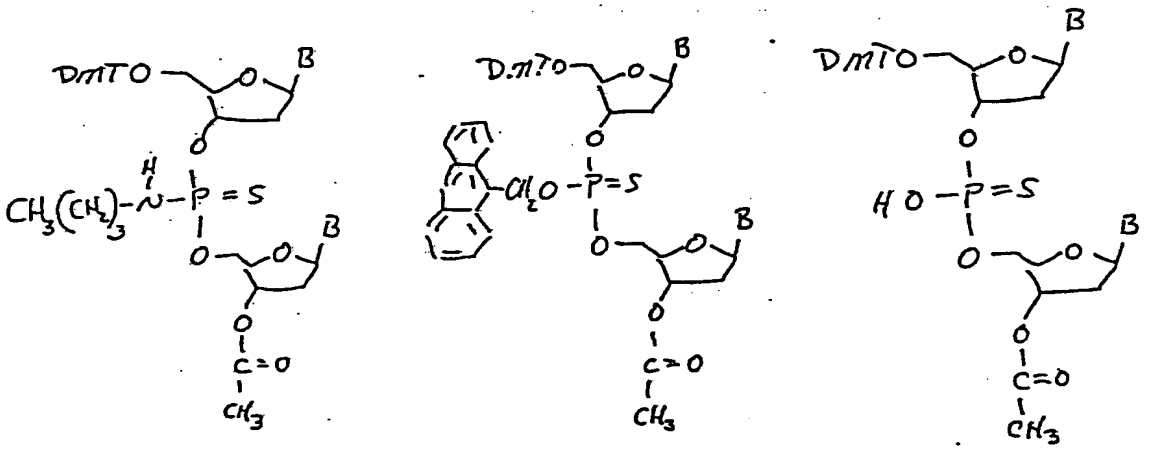
coupling efficiency). These syntheses were completed on silica based polymeric supports and in combination with phosphoramidite coupling methodologies (U.S. patents 4,458,066 and 4,415,732; also Science 230, 281-285, 1985). The oligodeoxynucleotides had the following sequences where the phosphorodithioate linkage in each segment is marked x and the normal phosphate internucleotide linkage is marked p.

d(TpGpTpGpGpApApTxTpGpTpGpApGpCpGpGpApTpApApCpApApTp-T)

d(ApApTpTpGpTpTpApTpCpCpGpCpTpCpApCpApApTxTpCpCpAp-CpA)

EXAMPLE VII

Synthesis of Dinucleoside Thioamidates, Thiotriesters, and Thioates of the formulae:



XIIa, P(Z,W) XIIb P(Z,V) XIIc P(Z,V)

where

B = 1-Thyminyl;

B = 1-(N-4-benzoylcytosinyl);

B = 9-(N-6-benzoyladeninyl);

B = 9-(N-2-isobutyrylguaninyl); and

DMT = dimethoxytrityl

The dinucleoside H-phosphonothioate was also found to be useful as a versatile synthon for preparing several analogs rapidly (5 min) in quantitative yield (^{31}P NMR). Thus, when oxidized with iodine/n-butylamine the phosphorothioamidate, XIIa,P(Z,W), was isolated in 92% yield. FAB$^-$ mass spectrum, 961 (M$^-$), 695 (DMT dT-3'-POSNHBU), 434 (5'-POSNHBU-dT-3'-OAc); ^{31}P NMR 74.4 and 74.0; ^{1}H NMR 8.36 and 8.34 (2 x s, NH), 7.59 and 7.56 (2 x s, H_6), 7.44-7.24 (m, aromatic), 6.82 (d, J = 8.7 Hz, DMT), 6.41 and 6.28 (m, H_1'), 5.28 and 5.23 (m, H_3'), 4.21 and 4.13 (m, H_4' (2 x) + H_5'), 3.77 (s, MeO-DMT), 3.43 (m, H_5'), 2.94 (m, CH_2-N), 2.41 (m, H_2'), 2.09 and 2.07 (2 x s,

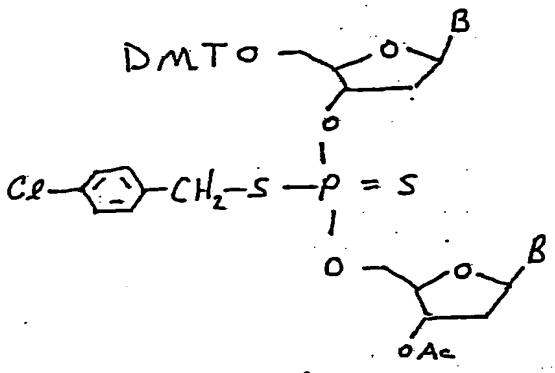
CH_3-acetyl), 1.93 and 1.88 (2 x s, CH_3-T), 1.42 (s, CH_3-T), 1.39-1.23 (m, CH_2), 0.90 and 0.83 (2 x t, J = 7.2 Hz and 7,1 Hz, CH_3). R_f = 0.56 (methanol/-dichloromethane, 1:9, v/v).

The dinucleoside H-phosphonothioate was converted quantitatively to a phosphorothioate triester by oxidation with iodine and 9-anthracenyl methanol (10 equivalents) under anhydrous conditions, XIIa, P(Z,V). FAB^+ mass spectrum, 527 (anhydro DMT dT): FAB^- mass spectrum, 906 (m-anthracenylmethyl), 639 (DMT dT-3'-PSO_2^-), 379 (5'-PSO_2^--dT-3'-OAc). ^{31}P NMR 51.7 and 51.0. R_f = 0.41 (methanol/dichloromethane, 1:9, v/v).

Treatment of the dinucleoside H-phosphonothioate with an aqueous solution of iodine and pyridine using art form conditions gave the dinucleoside phosphorothioate, XIIa,P(Z,V), in 87% yield. FAB^- mass spectrum, 906 (M^-), 603 (M-DMT), 379 (5'-PSO_2^-dT-3'-OAc) ^{31} P NMR 60.2 and 60.0.

EXAMPLE VIII

Synthesis of Dinucleoside Phosphorodithioate Triesters of the formula:



represented as XIIa, P(Z,Y), (Reaction Scheme C) where

B = 1-thyminyl;
B = 1-(N-4-toluoylcytosinyl);
B = 9-(N-6-benzoyladeninyl);
B = 9-(N2-isobutyrylguaninyl); and
DMT = dimethoxytrityl.
Ac = acetyl

and the further conversion of the deoxydicytidine derivative to deoxycytidine oligodeoxynucleotides having phosphorodithioate internucleotide linkages at various positions.

A. Synthesis of a Thymidine Dinucleotide Having a Phosphorodithioate Internucleotide Linkage.

5'-O-dimethoxytritylthymidine (1.2 g, 2.21 mmol) was dried by co-evaporation with anhydrous THF and then dissolved in THF (10 ml) and triethylamine (0.46 ml, 3.3 mmol). Bis(diisopropylamino) chlorophosphine (650 mg, 2.44 mmol) was added and the solution stirred at room temperature. After 35 minutes, the precipitate was removed by filtration and washed with

THF (1 ml). The combined filtrates containing the deoxynucleoside phosphorodiamidite were pooled, concentrated in vacuo, and redissolved in acetonitrile (5 ml). 3'-O-acetylthymidine (639 mg, 2.25 mmol) and tetrazole (142 mg, 2.0 mmol) were dried by co-evaporation with THF (10 ml), redissolved in acetonitrile (5 ml), and added to the acetonitrile solution of the deoxynucleoside phosphorodiamidite. After stirring for 45 minutes at room temperature, the reaction mixture was diluted with dichloromethane (75 ml), extracted with an aqueous sodium bicarbonate solution (5% w/v), dried over sodium sulfate, filtered, and concentrated in vacuo to a gum. The product was then purified by column chromatography (100 ml silica, ethylacetate:dichloromethane:-triethylamine; v/v/v) to yield 1.59 g of the deoxydinucleoside phosphoramidite (1.66 mmol, 75%). ^{31}P-NMR (CH_3CN 148.5, 148.1.

The deoxydinucleoside phosphoramidite was then converted to the deoxydinucleoside phosphorodithioate triester. The deoxydinucleoside phosphoramidite (1.59 g, 1.66 mmol) was dissolved in acetonitrile (7 ml). 4-Chlorobenzylmercaptan (1.0 ml, 1.20 g, 7.6 mmol) and tetrazole (281 mg, 4.01 mmol) were then added and the reaction mixture stirred at room temperature for 30 minutes. A solution of sulfur in toluene:2.6-lutidine (19:1, v/v, 10 ml containing 4 mmol atomic sulfur) was added and the resulting solution stirred for 10 minutes. The reaction mixture was diluted with ethylacetate (75 ml), extracted with an aqueous sodium bicarbonate solution (5%, w/v), dried over sodium sulfate, filtered and concentrated in vacuo to an oil. The oil was dissolved in ethylacetate (40 ml) and triturated with hexanes (200 ml) to give a crude product as a white powder. Purification by silica column chromatography (100 ml silica, 2-12% methanol in dichloromethane as %

eluant) yields the deoxydinucleoside phosphorodithioate triester (1.59 g, 1.52 mmol, 91%). ^{31}P-NMR ($CHCl_3$) 97.9, 96.4.

Removal of the 3'-O-acetyl group (0.15 M tert-butylamine in methanol, 0°C, 10 h) yields a deoxydinucleoside phosphorodithioate that can be used for DNA synthesis (1.26 g, 1.28 mmol, 84%) ^{31}P-NMR ($CHCl_3$) 97.3, 96.9. The deoxydinucleoside phosphorodithioate is converted to the 3'-phosphoramidite (see example V) and then used to synthesize DNA on a polymer support.

B. Synthesis of Deoxycytidine Oligomers Containing Phosphorodithioates

5'-O-Dimethoxytrityl-N-toluoyldeoxycytidine was prepared by minor modification of a published procedure (H. Koster, K. Kulinowski, T. Liese, W. Heikens, and V. Kohli, Tetrahedron 37, 363, 1981). Deoxycytidine hydrochloride (10 mmol, 2.64 g) was co-evaporated twice with anhydrous pyridine and resuspended in pyridine (50 ml). Trimethylchlorosilane (7.5 ml, 59 mmol) was added and the mixture stirred for 45 minutes at room temperature. o-Toluoyl chloride (1.44 ml, 11 mmol) was added and the reaction stirred for two additional hours. The reaction mixture was chilled in an ice bath, treated with methanol (10 ml) and 25% ammonium hydroxide (20 ml) for 30 min, and the suspension removed by filtration. The resulting solution was concentrated to dryness in vacuo. The resulting solid was suspended in 40 ml dichloromethane:methanol (8:2) and the insoluble salts removed by filtration. The filtrate was concentrated in vacuo to an oil, reconcentrated twice in vacuo after addition of pyridine and redissolved in pyridine (50 ml). After addition of 0.9 equivalents of dimethoxytrityl chloride (3.05 g), the reaction mixture was stirred for 30 min at 0°C and 30 min at room temperature. Dimethoxytritylchloride

(0.3 equivalents) was added and stirring was continued for 30 minutes. The reaction was quenched by addition of methanol (1 ml) and the solution concentrated in vacuo. The resulting oil was dissolved in dichloromethane (75 ml) and extracted sequentially with aqueous 5% sodium bicarbonate (w/v) and brine. The combined organic phase was dried over sodium sulfate, filtered, concentrated to dryness in vacuo, dissolved in dichloromethane:pyridine (99.5:0.5, v/v) and the product purified by column chromatography (50 g silica, dichloromethane:methanol:pyridine gradient from 0 to 3% methanol; 400 ml each). Fractions containing 5'-O-dimethoxytrityl-N-toluoyldeoxycytidine were pooled, concentrated in vacuo, redissolved in ethylacetate and precipitated into pentane (5.01 g, 7.7 mmol, 77%).

3'-O-Phenoxyacetyl-N-toluoyldeoxycytidine was prepared by minor modification of a published procedure (C. B. Reese and J. C. M. Stewart, Tetrahedron Letters 4273, 1968). 5'-O-Dimethoxytrityl-N-toluoyldeoxycytidine (1.94 g, 3 mmol) and phenoxyacetic anhydride (1.72 g, 6 mmol) was dissolved in tetrahydrofuran (50 ml). After addition of pyridine (0173 ml, 9 mmol), the solution was stirred for 14 hours at room temperature and then concentrated in vacuo. The resulting oil was dissolved in dichloromethane (75 ml), extracted twice with 5% aqueous sodium bicarbonate (100 ml, w/v) and the combined aqueous phases extracted with dichloromethane (50 ml). The product in the combined organic phase was dried over sodium sulfate, filtered, concentrated to dryness in vacuo, redissolved in ethylacetate and precipitated in pentane. The solid corresponding to totally protected deoxycytidine was dissolved in dichloromethane:methanol (8:2, v/v) and chilled in an ice bath. A solution of

p-toluenesulfonic acid (2.28 g, 12 mmol) in dichloromethane:methanol (50 ml, 8:2, v/v) was added and the solution stirred for one hour in an ice bath. The reaction was then quenched by addition of 5% aqueous sodium bicarbonate. The organic layer was extracted with brine and the aqueous phase re-extracted with dichloromethane (60 ml). The combined organic phase was dried over sodium sulfate, filtered and concentrated to dryness in vacuo. The resulting oil was dissolved in dichloromethane and the product purified by silica gel column chromatography (20 g of silica, elution with dichloromethane and dichloromethane:methanol (1 to 3% methanol). Fractions containing 3'-O-phenoxyacetyl-N-toluoyldeoxycytidine were pooled, concentrated to an oil, and the product isolated as a precipitate by addition of ethylacetate (1.20 g, 83%).

Deoxydicytidine phosphoroamidite in protected form was prepared using the following procedure. 5'-O-Dimethoxytrityl-N-toluoyldeoxycytidine (647 mg, 1 mmol) was co-evaporated three times with THF, dissolved in THF (5 ml) and triethylamine (0.21 ml, 1.5 mmol) and reacted with bis(N,N-diisopropylamino) chlorosphosphine (320 mg, 1.2 mmol). After 90 minutes under argon, the reaction mixture was filtered under argon pressure to remove insoluble salts. The salts were washed with THF (2 ml). The filtrate was concentrated to dryness and the product redissolved in acetonitrile (2 ml). 3'-O-Phenoxyacetyl-N-toluoyldeoxycytidine (527 mg, 1.1 mmol) and tetrazole (70 mg, 1 mmol) were suspended in acetonitrile (4 ml) and the above solution, including 1.5 ml acetonitrile used to wash the flask, was added. The reaction mixture was stirred under argon for 105 min. and then poured into ethylacetate:triethylamine (99:1, V/V, 50ml). After two extractions with 2M triethylammonium bicarbonate (20 ml each) and back

extraction of the aqueous phase with ethylacetate:triethylamine (99:1,v/v, 25 ml), the organic phase was dried over sodium sulfate, filtered, and concentrated in vacuo. Purification was achieved by silica gel column chromatography (25 g silica, elution with hexanes:dichloromethane:triethylamine; 50:50:0.5, 400 ml; 45:55:0.5, 200 ml; 40:60:0.5, 200 ml; and 35:65:0.5, 100 ml). Product fractions were pooled, concentrated in vacuo, and precipitated into pentane (67%).

^{31}P-NMR ($CHCl_3$) 149.3, 149.1. ^{1}H-NMR 8.22 and 8.19 (s, H6), 7.54-6.80 (m, HAr), 6.30 (m, H1'), 5.39 (m, H3'), 4.67 (m, CH_2 phenoxyacetyl + H iPr), 4.25 (m, H4'), 3.78 (2s, Me DMT), 3.5 (m, H5', 5''), 2.8 and 2.3 (m, H2', 2''), 2.47 (m, Me tol), 1.14 (m, Me iPr).

Deoxydicytidine phosphorodithioate was prepared using the following procedure. The deoxydicytidine phosphoramidite as prepared in the previous procedure (1.40 g, 1.12 mmol) was dissolved in acetonitrile (5 ml) (previously flushed with helium to avoid oxygen oxidation of thiophosphite) and 4-chlorobenzylmercaptan (0.5 ml, 3.7 mmol) and tetrazole (190 mg, 2.7 mmol) were added. The solution was stirred under argon for 30 min and, without isolation, the resulting thiophosphite (completely formed in 15 minutes as shown by ^{31}P-NMR,

193.4 ppm in the crude reaction mixture) was oxidized to the phosphorodithioate triester by addition of 5 ml of a 0.4 M solution of sulfur in toluene:lutidine (19.1, v/v). Based on ^{31}P-NMR analysis (δ 94.9, 94.7), oxidation was complete after 10 minutes. The reaction mixture was diluted with ethylacetate (75 ml), extracted twice with 5% aqueous sodium bicarbonate (75 ml each), and the combined aqueous phases back extracted with

ethylacetate (50 ml). The combined organic phases were dried over sodium sulfate, filtered, and concentrated in vacuo to an oil. The oil was dissolved in a minimal amount of dichloromethane, diluted with ethylacetate to approximately 40 ml, and the product precipitated by addition of 200 ml hexanes. The white precipitate was filtered, redissolved in dichloromethane, and the solution concentrated to dryness. The product was purified by silica gel column chromatography (40 g silica gel, elution with dichloromethane:hexanes:triethylamine, 66:33:0.03, 400 ml and dichloromethane:triethylamine, 100:0.03,200 ml). Fractions containing the completely protected product were pooled, concentrated in vacuo, redissolved in dichloromethane, and precipitated into pentane (60%).

^{31}P-NMR ($CHCl_3$) 97.5, 96.7. ^{1}H-NMR 8.1 (m, H6), 7.6-6.8 (m, HAr), 6.25 (m, H1'), 5.25 (m, H3'), 4.70 (m, CH_2 phenoxyacetyl), 4.5-4.0 (m, CH_2 benzyl, H5', H4'), 3.79 (s, Me DMT), 3.73-3.35 (m, H5'), 3.0-2.55 and 2.45-1.95 (m, H2', 2''), 2.50 (m, Me tol).

The 3'-O-phenoxyacetyl protecting group was removed using the following procedure. The completely protected deoxydicytidine phosphorodithioate triester (355 mg, .264 mmol) was dissolved in acetonitrile (3 ml) and diluted with methanol (9 ml). After chilling the solution in an ice bath, tert-butylamine in methanol (0.3 M, 12 ml) was added and the reaction mixture stirred for 90 min in an ice bath. The reaction solution was concentrated to dryness and the product purified by silica gel column chromatography (30 g silica, elution with dichloromethane:triethylamine, 100:0.03, 100 ml followed by 200 ml each of dichloromethane:methanol:-triethylamine, 99:1:0.03, 98:2:0.03 and 97:3:0.03). Product fractions were concentrated to dryness,

redissolved in dichloromethane, and precipitated into pentane (95% yield).

^{31}P-NMR ($CDCl_3$) 96.5, 96.2. ^{1}H-NMR 8.2-8.06 (m, H6), 7.52-6.81 (m, HAr), 6.25 (m, H1'), 5.24 (m, H3'), 4.5-4.0 (m, CH_2 benzyl, H3', H4', H5'), 3.79 (s, Me DMT), 3.6-3.3 (m, H5'), 2.95-2.55 and 2.45-2.05 (m, H2', 2''), 2.50 (m, Me tol).

The deoxydicytidine phosphorodithioate was next converted to the 3'-phosphoramidite which is useful as a synthon for synthesizing DNA containing dithioate internucleotide linkages. The deoxydicytidine phosphorodithioate having a free 3'-hydroxyl (304 mg, 0.251 mmol) was dissolved in acetonitrile (5 ml). Bis(diisopropylamino)-β-cyanoethoxyphosphine (121 mg, 0.402 mmol) and tetrazole (20 mg, 0.286 mmol) were added under argon and the solution stirred for 2 hours. After quenching with ethylacetate:triethylamine (19.5:0.5) and diluting further with ethylacetate (20 ml), the reaction mixture was extracted twice with 2 M triethylammonium bicarbonate (13 ml each) and the aqueous phase back extracted with ethylacetate: triethylamine (19.5:0.5). The organic layer was dried over sodium sulfate, filtered, and concentrated to an oil *in vacuo*. The resulting oil was redissolved in dry ethylacetate and precipitated into pentane (87% yield).

^{31}P-NMR (dichloromethane) 149.5, 149.2, 149.0, 96.5, 96.0.

Deoxycytidine pentadecamers containing phosphorodithioate internucleotide linkages at selected sites were synthesized using the deoxydicytidine phosphorodithioate synthons having a 3'-O-(β-cyanoethyl)-N,N-diisopropylphosphoramidite moiety as described above and 5'-O-dimethoxytrityl-N-benzoyldeoxycytidine -3'-O-(β-cyanoethyl)-N,N-diisopropylphosphoramidite. The standard

phosphoramidite synthesis methodology was used (M. H. Caruthers and S. L. Beaucage, U. S. Patent 4,415,732 and M. H. Caruthers and M. D. Matteucci, U. S. Patent 4, 458,066). The average coupling efficiency was 99% (3 minute coupling time, 0.2 mol deoxycytidine on controlled pore glass as a support). The products were freed of protecting groups by treatment with a solution of thiophenol:triethylamine:dioxane (1:1:2, v/v/v) at room temperature for 6 hours (some product remains as the S-protected dithioate (5-10%) when analyzed by gel electrophoresis and concentrated ammonium hydroxide at 55°C (15 hours). Purification of the final product was by either polyacrylamide gel electrophoresis or high performance liquid chromatography. Three pentadecamers having phosphorodithioate linkages at specific positions were synthesized and have the following sequence:

d(CpCxCpCpCpCpCpCpCpCpCpCxCpC)

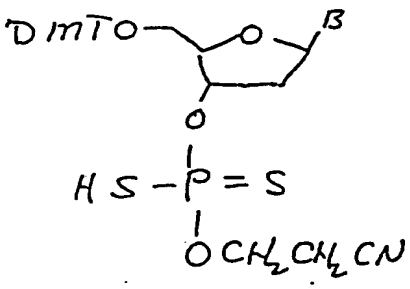
d(CpCpCpCpCpCpCxCpCpCpCpCpCpC)

d(CxCpCxCpCxCpCxCpCxCpCxCpCxCpC)

where x represents a dithioate linkage and p represents the natural internucleotide linkage.

EXAMPLE IX

Synthesis of Nucleoside 3'-Phosphorodithioate of the formula:



represented as XVIa:

B = 1-Thyminyl;

B = 1-(N-4-benzoylcytosinyl);

B = 9-(N-6-benzoyladeninyl);

B = 9-(N-2-isobutyrylguaninyl); and

DMT = dimethoxytrityl

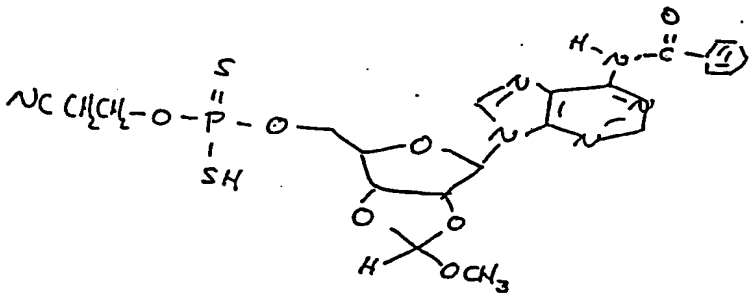
3'-O-(Diisopropylamino)-2-cyanoethylphosphino--5'-O'(di-p-methoxytrityl) thymidine (27.7 mg, 0.04 mmol) was prepared by art form methods (M. H. Caruthers and S. L. Beaucage U.S. Patent 4,415,732) and then dissolved in anhydrous acetonitrile (440 1). Hydrogen sulfide was bubbled through for 1 min and tetrazole (7.0 mg in 220 1 CH_3CN, 0.2 mmol) was added. After 10 min ^{31}P NMR spectroscopy showed quantitative conversion to the two diastereomers (70.9 and 70.2 ppm, $^{1}J_{PH}$ = 675 Hz) of the nucleoside H-phosphonothioate. Excess of elementary sulfur converted the H-phosphonothioate in quantitative yield within 1/2 h under stirring at rt to the nucleoside 3'-phosphorodithioate. ^{31}P NMR (CH_3CN) 114.0 ppm. FAB$^-$ 708 (M^-), 182 (M-DMTdT+O). ^{1}H NMR ($CDCl_3$) 7.53 (s, H_6), 7.35-6.81 (m aromatic), 6.15 (t, H_1', J = 6.4 Hz), 5.12 (m, H_3'), 4.20 (m, H_5'), 3.95 (m, H_4'), 3.18 (s, MeO-DMT), 3.47 (m, CH_2O-P),

2.77 (t, CH_2CN, J = 6.2 Hz), 2.56-2.44 (m, H_2'), 1.91 (s, CH_3-T).

Protected nucleoside 3'-phosphorodithioate was dissolved in 80% aqueous acetic acid (4 ml) and left for 30 min at rt. The reaction mixture was then diluted with water (4 ml) and extracted 3 times with ether (5 ml). The water phase was evaporated to an oil followed by a co-evaporation with water (5 ml). The oil was redissolved 25% aqueous ammnia and incubated ag 55°C for 16 h. The mixture was re-evaporated and lyophilized with water to yield the nucleoside 3'-phorphorodithioate. FAB^- 338 (M^-). FAB+ 338 ($dT\text{-}P^+_{SH}$ = S).

EXAMPLE X

Synthesis of Nuceloside 5'-Phosphorodithioate of the formula:



represented as compound XVIa where

B = 1-Uracilyl;

B = 1-(N-4-benzoylcytosinyl);

B = 9-(N-6-benzoyladeninyl);

B = 9-(N-2-isobutyrylguaninyl).

A solution of N^6-benzoyl-2-3-methoxymethylidene-adenosine (413 mg, 1.1 mmol) in anhydrous $CHCl_3$ (5 ml) and tetrazole (76 mg, 1.1 mmol, in CH_3CN (2.2 ml) was added 2-cyanoethyl-N,N,N',N'-tetraisopropyl phosphorodiamidite (345 mg, 1.1 mmol) and stirred at rt for 20 min. Precipitation of the ammonium tetrazolide appeared after 1/2 min. The reaction mixture was diluted with CH_2Cl_2 (50 ml) and extracted with $NaHCO_3$ (5% w/v, 50 ml), back-extracted with CH_2CL_2 (25 ml), the organic phase dried over Na_2SO_4, filtered and evaporated to dryness <u>in vacuo</u>. ^{31}P NMR analysis (CH_3CN) showed 147.9 ppm. Crude product (0.71 g) was dissolved in anhydrous CH_3CN (5 ml) and bubbled with hydrogen sulfide for one min. Tetrazole (175 mg, 2.5 mmol in CH_3CN (5ml) was added and again

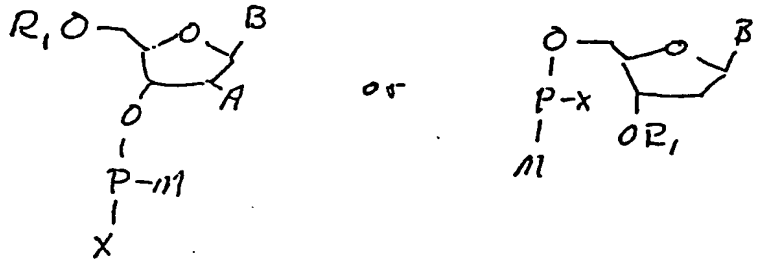
hydrogen sulfide was bubbled through the reaction mixture for 1 min. The reaction mixture was sealed and after 10 min a precipitate of ammonium tetrazolide appeared (^{31}P NMR (CH_3CN) 72.2 and 71.8 ppdm., $^{1}J_{PH}$ = 669 Hz). The reaction mixture was evaporated to an oil in vacuo, redissolved in ethylacetate (50ml), extracted with TEAB (1 M, pH = 7.4, 50ml), and back-extracted with ethylacetate (50ml). The combined organic phases were dried over Na_2SO_4, filtered, evaporated, and the oil was redissolved in CH_2Cl_2 (5ml). Excess elementary sulfur (80 mg, 2.5 mmol, in 5 ml toluene/2,6-lutidine, 19:1m v/v) was added. Stirring at room temperature for 1 h gave the phosphorodithioate product. ^{31}P NMR (CH_3CN) 114.4 and 114.3. R_f (silica) = 0.34 in CH_2Cl_2 (9:1, v/v).

Thus while we have illustrated and described the preferred embodiment of our invention, it is to be understood that this invention is capable of variation and modification and we therefore do not wish to be limited to the precise terms set forth, but desire to avail ourselves of such changes and alterations which may be made for adapting the invention to various usages and conditions. Accordingly, such changes and alterations are properly intended to be within the full range of equivalents, and therefore within the purview of the following claims.

Having thus described our invention and the manner and process of making and using it in such full, clear, concise, and exact terms so as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same;

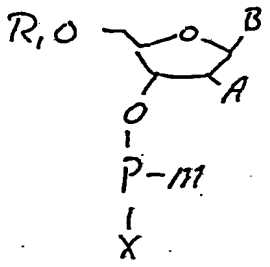
We claim:

1. A compound according to the formula:



wherein B is a nucleoside or deoxynucleoside base; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide, and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; R_1 is a blocking group; X is a secondary amino group of the formula NR_6R_7, wherein R_6 and R_7 taken separately each represent a heteroatom substituted or unsubstituted alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, aralkenyl, cycloalkenyl, alkenyl, aralkynyl, cycloalkynyl or alkynyl, R_6 and R_7 when taken together form an alkylene chain containing up to 5 carbon atoms in the principal chain and a total of up to 10 carbon atoms with both terminal valence bonds of the chain being attached to the nitrogen atom to which R_6 and R_7 are attached, and when R_6 and R_7 are taken together with the nitrogen atom to which they are attached form a nitrogen heterocycle including at least one additional heteroatom from the group of nitrogen, oxygen, and sulfur; and M is sulfur single bonded to phosphorus and to R_8 where R_8 is a heteroatom substituted or unsubstituted alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, aralkenyl, cycloalkenyl, alkenyl, alkynyl, aralkynyl or cycloalkynyl.

2. A compound according to the formula:



wherein B is a nucleoside or deoxynucleoside base, A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; R_1 is a blocking group; X is a secondary amino group of the formula NR_6R_7, wherein R_6 and R_7 taken separately each represent a heteroatom substituted or unsubstituted alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, aralkenyl, cycloalkenyl, alkenyl, aralkynyl, cycloalkynyl or alkynyl, R_6 and R_7 when taken together form an alkylene chain containing up to 5 carbon atoms in the principal chain and a total of up to 10 carbon atoms with both terminal valence bonds of the chain being attached to the nitrogen atom to which R_6 and R_7 are attached, and when R_6 and R_7 are taken together with the nitrogen atom to which they are attached form a nitrogen heterocycle including at least one additional heteroatom from the group of nitrogen, oxygen, and sulfur; and M is sulfur single bonded to phosphorus and to R_8 where R_8 is a heteroatom substituted or unsubstituted alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, aralkenyl, cycloalkenyl, alkenyl, alkynyl, aralkynyl or cycloalkynyl.

3. A compound according to claim 1 or 2 wherein R_1 is a trityl group, a di-p-anisylphenylmethyl group or a p-anisyldiphenylmethyl group.

4. A compound according to claim 1 or 2 wherein R_8 is benzyl, a substituted benzyl, 2,4-dichlorobenzyl, a lower alkyl, a heteroatom substituted lower alkyl, or β-cyanoethyl.

5. A compound according to Claim 4 wherein M is sulfur single bonded to phosphorus and R_8.

6. A compound according to claim 1 or 2 wherein X is a secondary amino group, NR_6R_7.

7. A compound according to claim 6 where X is diisopropylamino, dimethylamino, diethylamino, dibutylamino, and pyrrolidinyl.

8. A compound according to claim 1 or 2 wherein B is adenine, guanine, cytosine, uracil, and thymine.

9. The compound according to claim 2 wherein R_1 is di-p-anisylphenylmethyl, B is thyminyl, M is 2,4-dichlorobenzylmercaptyl, A is H, and X is dimethylamino.

10. The compound according to claim 2 wherein R_1 is di-p-anisylphenylmethyl, B is 9-(N-6-benzoyladeninyl), M is 2,4-dichlorobenzylmercaptyl, A is H, and X is dimethylamino.

11. The compound according to claim 2 wherein R_1 is di-p-anisylphenylmethyl, B is 1-(N-4-benzoylcytosinyl), M is

2,4-dichlorobenzylmercaptyl, A is H, and X is dimethylamino.

12. The compound according to claim 2 wherein R_1 is di-p-anisylphenylmethyl, B is 9-(N-2-isobutyrylguaninyl), M is 2,4-dichlorobenzylmercaptyl, A is H, and X is dimethylamino.

13. A compound according to claim 1 wherein X is selected from the class consisting of dimethylamino, diethylamino, diisopropylamino, dibutylamino, methylpropylamino, methylhexylamino, methylcyclohexylamino, ethylcyclopropylamino, methylbenzylamino, methylphenylamino, ethylchloroethylamino, methyltoluylamino, methyl-p-chlorophenylamino, methylcyclohexylmethylamino, bromobutylcyclohexylamino, methyl-p-cyanophenylamino, ethyl-β-cyanoethylamino, morpholino, thiomorpholino, pyrrolidino, piperidino, 2,6-dimethylpiperidino and piperazino.

14. A compound according to claim 1 wherein X is dimethylamino.

15. A compound according to claim 2 wherein X is selected from the class consisting of dimethylamino, diethylamino, diisopropylamino, dibutylamino, methylpropylamino, methylhexylamino, methylcyclohexylamino, ethylcyclopropylamino, methylbenzylamino, methylphenylamino, ethylchloroethylamino, methyltoluylamino, methyl-p-chlorophenylamino, methylcyclohexylmethylamino, bromobutylcyclohexylamino, methyl-p-cyanophenylamino, ethyl-β cyanoethylamino, morpholino, thiomorpholino,

pyrrolidino, piperidino, 2,6-dimethylpiperidino and piperazino.

16. A compound according to claim 2 wherein X is dimethylamino.

17. A compound according to claim 1 where M is selected from a class consisting of ethylmercaptyl, methylmercaptyl, propylmercaptyl, butylmercaptyl, β-cyanoethylmercaptyl, benzylmercaptyl, 4-chlorophenylmercaptyl, 4-chlorobenzylmercaptyl, 2,4-dichlorobenzylmercaptyl, cyclohexylmercaptyl, and 4-nitrophenylethylmercaptyl.

18. A compound according to claim 1 where M is 2,4-dichlorobenzylmercaptyl.

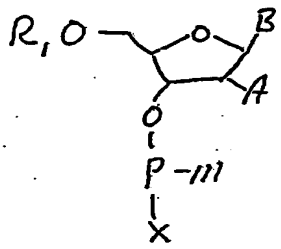
19. A compound according to claim 2 where M is selected from a class consisting of ethylmercaptyl, methylmercaptyl, propylmercaptyl, butylmercaptyl, β-cyanoethylmercaptyl, benzylmercaptyl, 4-chlorophenylmercaptyl, 4-chlorobenzylmercaptyl, 2,4-dichlorobenzylmercaptyl, cyclohexylmercaptyl, and 4-nitrophenylethylmercaptyl.

20. A compound according to claim 2 where M is 2,4-dichlorobenzylmercaptyl.

21. A process for production of oligonucleotides which comprises the step of condensing the 3'-OH or 5'-OH group of nucleoside or oligonucleotide by a coupling agent through the 5'-O- or 3'-O-, respectively, of said nucleoside or oligonucleotide, with a compound according to claim 1 or 2.

22. A process for production of oligonucleotides which comprises the step of condensing the 5'-OH

group of a nucleoside of oligonucleotide by a coupling agent through the 5'-O-, respectively, of said nucleoside or oligonucleotide with a compound according to claim 2 of the following formula:



wherein R_1 is a blocking group; B is a nucleoside or deoxynucleoside base; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; X is NR_6R_7, and M is sulfur single bonded to phosphorus and to R_8 where R_6, R_7 and R_8 are as previously defined.

23. A process according to claim 22 wherein B is adenine, guanine, cytosine or thymine and A is H.

24. A process according to claim 22 wherein X is dimethylamino.

25. A process according to claim 22 where M is 2,4-dichlorobenzylmercaptyl.

26. A process according to claim 22 where the nucleoside or oligonucleotide having a free 5'-OH group is linked to a polymer support.

27. A process according to claim 22 where the coupling agent is tetrazole, substituted tetrazole, tetrazolide salts, substituted tetrazolide salts and amine salts.

28. A process according to claim 22 including the further step of oxidizing the resulting thiophosphite triester to a phosphorodithioate triester.

29. A process according to claim 28 wherein elementary sulfur is the oxidizing agent.

30. A process according to claim 22 including the further step of oxidizing the thiophosphite triester to a phosphorothioate.

31. A process according to claim 30 wherein t-butylhydroperoxide is the oxidizing reagent.

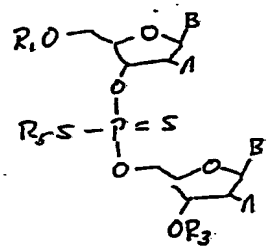
32. A repetitive process whereby the compound of claims 1 or 2 is used to produce oligonucleotides having phosphorodithioate and phosphorothioate internucleotide linkages in any and all combinations and as oligonucleotides having only phosphorodithioate or phosphorothioate linkages.

33. Oligonucleotides in protected or unprotected form having only phosphorodithioate or phosphorothioate internucleotide linkages.

34. A repetitive process whereby the compound of claims 1 or 2 is used in combination with nucleoside phosphoramidites, nucleoside phosphate diesters, nucleoside phosphates, or nucleoside H-phosphonates to form oligonucleotides having combinations of natural phosphate, phosphorodithioate, phosphorothioate and phosphoroamidate internucleotide linkages whereby combinations of these linkages are produced by oxidation of the appropriate thiophosphite, H-phosphonate or phosphite with t-butylhydroperoxide, sulfur, or iodine in combination with water or amines.

35. Oligonucleotides in protected or unprotected form having any and all combinations of natural phosphate, phosphorothioate, phosphorodithioate and phosphoroamidate internucleotide linkages wherein at least one of these internucleotide linkages is phosphorodithioate or phosphorothioate.

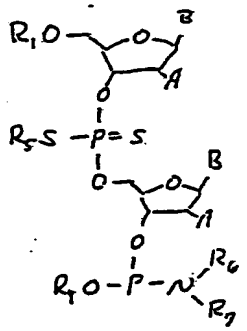
36. A compound of the formula:



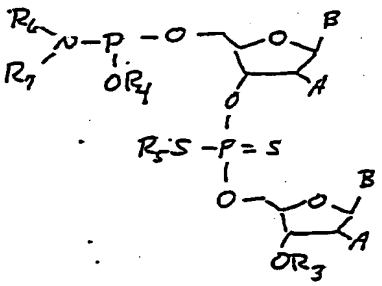
wherein B is a nucleoside or deoxynucleoside base; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; R_1 and R_3 are blocking groups; and R_5 is a blocking group.

37. A compound according to claim 36 wherein R_1 is di- p -anisylphenylmethyl, R_3 is acetyl, levulinyl, phenoxyacetyl or other blocking group and R_5 is 2,4-dichlorobenzylmethyl or β -cyanoethyl, A is H, and B is a deoxynucleoside base.

38. A compound of the formula:

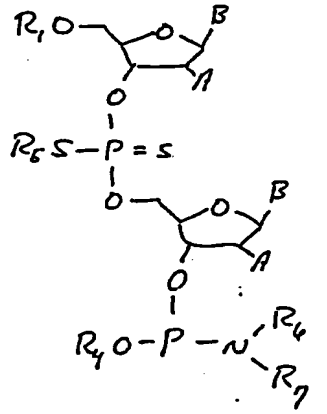


or



wherein B is a nucleoside or deoxynucleoside base; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; R_1, R_3, R_4 and R_5 are blocking groups, and R_6 and R_7 are heteroatom substituted or unsubstituted alkyl, aryl or aralkyl substituents.

39. A compound of the formula:



wherein B is a nucleoside or deoxynucleoside base; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; R_1, R_4 and R_5 are blocking groups, and R_6 and R_7 are heteroatom substituted or unsubstituted alkyl, aryl or aralkyl substituents.

40. A process for production of oligonucleotides which comprises the step of condensing the 3'-OH or 5'-OH group of a nucleoside or oligonucleotide by a coupling agent through the 5'-O- or 3'-O-, respectively, of said nucleoside or oligonucleotide, with a compound according to claim 38 or 39 followed by oxidation to pentavalent phosphorus.

41. A process for production of oligonucleotides which comprises the step of condensing the 5'-OH

group of a nucleoside or oligonucleotide by a coupling agent through the 5'-O- respectively of said nucleoside or oligonucleotide with a compound according to claim 39 followed by oxidation to pentavalent phosphorus.

42. A process of claim 41 wherein B is adenine, guanine, cytosine or thymine, and A is H.

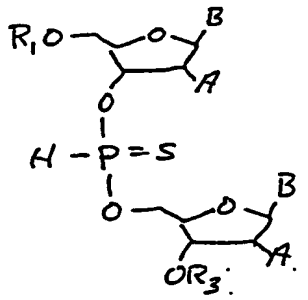
43. A process according to claim 41 wherein NR_6R_7 is diisopropylamino, R_4 is methyl or β-cyanoethyl, R_5 is methyl or 2,4-dicholorobenzyl or β-cyanoethyl, and R_1 is di- p -anisylphenylmethyl.

44. A process according to claim 41 where the nucleoside or oligonucleotide having a free 5'-OH group is linked to a polymer support and the synthesis is repeated many times to form an oligonucleotide of defined sequence.

45. An oligonucleotide in blocked or unblocked form and containing phosphorodithioate linkages.

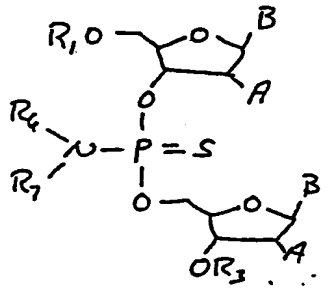
46. An oligonucleotide in protected or unprotected form and containing a phosphorodithioate linkage that is produced from a compound of claim 36 wherein B is a nucleoside or deoxynucleoside base, A is K or KR_2 where K is OH, H halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; R_5 is a blocking group, R_1 is H or a blocking group, and R_3 is H or a blocking group.

47. A compound of the formula:



wherein B is a nucleoside or deoxynucleoside base; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; and R_1 and R_3 are blocking groups.

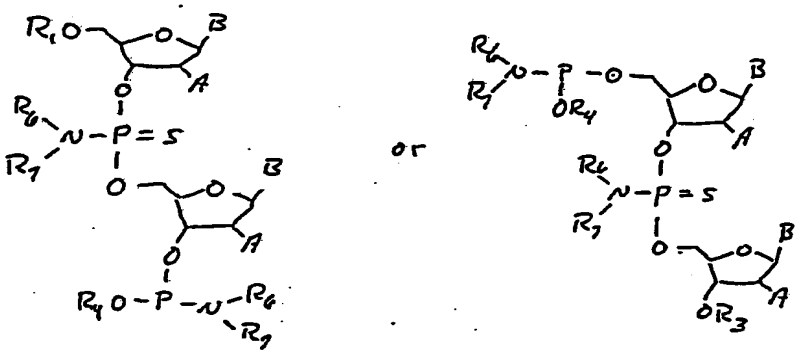
48. A compound of the formula:



wherein B is a nucleoside or deoxynucleoside base; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; and R_1 and R_3 are blocking groups;

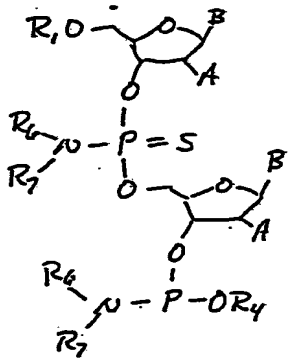
and R_6 and R_7 are substituted or unsubstituted alkyl, aryl, or aralkyl substituents.

49. A compound of the formula:



wherein B is a nucleoside or deoxyoligonucleotide base; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; and R_1, R_3 and R_4 are blocking groups; and R_6 and R_7 are substituted or unsubstituted alkyl, aryl, or aralkyl substituents.

50. A compound of the formula:



wherein B is a nucleoside or deoxynucleoside base; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted

blocking group; and R_1 and R_4 are blocking groups; and R_6 and R_7 are substituted or unsubstituted alkyl, aryl, or aralkyl substituents.

51. A process for production of oligonucleotides which comprises the step of condensing the 3'-OH or 5'-OH group of a nucleoside or oligonucleotide by a coupling agent through the 5'-O- or 3'-O-, respectively, of said nucleoside or oligonucleotide, with a compound according to claim 49 or 50 followed by oxidation to pentavalent phosphorus.

52. A process for production of oligonucleotides which comprises the step of condensing the 5'-OH group of a nucleoside or oligonucleotide by a coupling agent through the 5'-O-, respectively, of said nucleoside or oligonucleotide with a compound according to claim 50 followed by oxidation to pentavalent phosphorus.

53. A process of claim 52 wherein B is adenine, guanine, cytosine, or thymine and A is H.

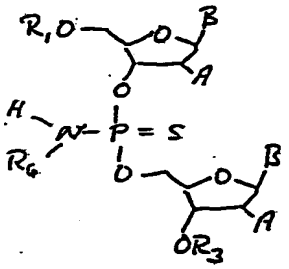
54. A process according to claim 52 wherein NR_6R_7 attached to trivalent phosphorus is diisopropylamine, R_4 is methyl or β-cyanoethyl, and R_1 is di-p-anisylphenylmethyl.

55. The process according to claim 52 where the nucleoside or oligonucleotide having a free 5'-OH is linked to a polymer support and the synthesis is repeated many times to form an oligonucleotide of defined sequence.

56. An oligonucleotide in blocked or unblocked form and containing phosphorothioamidate linkages produced by the process of claim 52.

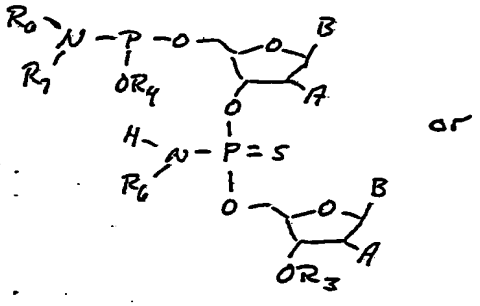
57. An oligonucleotide in blocked or unblocked form and containing a phosphorothioamidate linkage produced from a compound of claim 48 wherein B is a nucleotide or deoxynucleoside base, A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; R_1 is H or a blocking group, R_3 is H or a blocking group, and R_6 and R_7 are heteroatom substituted or unsubstituted alkyl, aryl or aralkyl groups.

58. A compound of the formula:

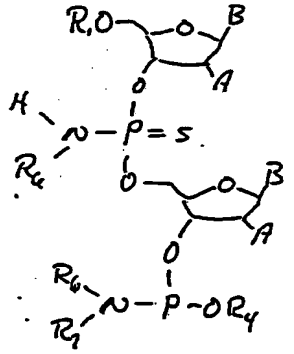


wherein B is a nucleoside or deoxynucleoside base; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; and R_1 and R_3 are blocking groups; and R_6 is a substituted or unsubstituted alkyl, aryl, or aralkyl substituent.

59. A compound of the formula:

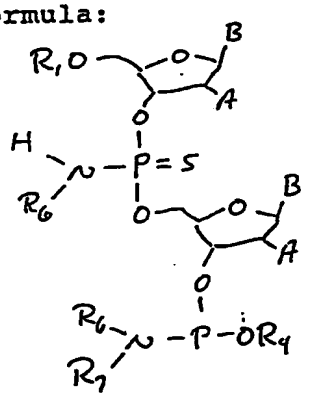


or



wherein B is a nucleoside or deoxynucleoside base; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; and R_1, R_3 and R_4 are blocking groups; and R_6 and R_7 are substituted or unsubstituted alkyl, aryl, or aralkyl substituents.

60. A compound of formula:



wherein B is a nucleoside or deoxynucleoside base; A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; and R_1 and R_4 are blocking groups; and R_6 and R_7 are substituted or unsubstituted alkyl, aryl, or aralkyl substituents.

61. A process for production of oligonucleotides which comprises the step of condensing the 3'-OH or 5'-OH group of a nucleoside or oligonucleotide by a coupling agent through the 5'-O- or 3'-O-, respectively, of said nucleoside or oligonucleotide, with a compound according to claim 59 or 60 followed by oxidation to pentavalent phosphorus.

62. A process for production of oligonucleotides which comprises the step of condensing the 5'-OH

group of a nucleoside or oligonucleotide by a coupling agent through the 5'-O-, respectively, of said nucleoside or oligonucleotide with a compound according to claim 60 followed by oxidation to pentavalent phosphorus.

63. A process of claim 62 wherein B is guanine, adenine, cytosine or thymine and A is H.

64. A process according to claim 62 wherein NR_6R_7 attached to trivalent phosphorus is diisopropylamine, R_4 is methyl or β-cyanoethyl and R_1 is di-p-anisylphenylmethyl.

65. A process according to claim 62 where the nucleoside or oligonucleotide having a free 5'-OH is linked to a polymer support and the synthesis is repeated many times to form an oligonucleotide of defined sequence.

66. An oligonucleotide in blocked or unblocked form and containing phosphorothioamidate linkages produced by the process of claim 62.

67. An oligonucleotide in blocked or unblocked form and containing a phosphorothioamidate linkage that is produced from a compound of claim 58 wherein B is a nucleoside or deoxynucleoside base, A is K or KR_2 where K is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; R_1 is H or a blocking group, R_3 is H or a blocking group, and R_6 is a heteroatom substituted or unsubstituted alkyl, aryl or aralkyl group.

68. The process of converting a compound of claim 47 to a phosphorothioate triester by oxidation with R_4OH

and an oxidizing agent where R_4 is a heteroatom substituted or unsubstituted alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, alkenyl, cycloalkenyl, aralkenyl, alkynyl, aralkynyl, or cycloalkynyl substituents.

69. A process of claim 68 where the oxidizing agent is I_2 and R_4OH and R_4 is anthracenylmethyl.

70. A process of converting a compound of claim 47 to a phosphorothioate diester by oxidation.

71. Oligonucleotides containing phosphorothioate diesters when produced by the process of claim 70.

72. A process of producing oligonucleotides from the compound of claim 36 by using H-phosphonates, a phosphate monoester, or phosphate as R_3 or R_1.

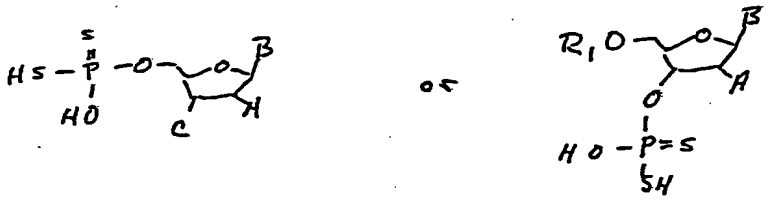
73. Oligonucleotides containing phosphorodithioates.

74. A process of producing oligonucleotides from the compound of claim 48 by using H-phosphonate, a phosphate monoester or phosphate as R_3 or R_1.

75. Oligonucleotides containing phosphorothioamidates.

76. A process of producing oligonucleotides from the compound of claim 58 by using H-phosphonate, a phosphate monoester, or phosphate as R_3 or R_1.

77. A compound represented by the following formula:



wherein B is a nucleoside or deoxynucleoside base, A is K or KR_2 where is OH, H, halogen, SH, NH_2 or azide and KR_2 is oxygen, sulfur or nitrogen as K and R_2 is a heteroatom substituted or unsubstituted blocking group; and R_1 is H or a blocking group, C is H, OH or OR_3 where R_3 is a blocking group.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02293

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.[4]: C07H 17/00
U.S. CL. 536/27, 28, 29; 538/18

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. CL.	536/27, 28, 29; 538/18

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	US, A, 3,846,402 (ECKSTEIN) 5 NOVEMBER 1984, SEE ABSTRACT.	1-77
A	US, A, 3,451,997 (FUJIMOTO) 24 JUNE 1969 SEE COLUMNS 1-2	1-77
A	US, A, 3,853,844 (SHUMAN) 12 DECEMBER 1974 SEE COLUMN 1.	1-77
A	US, A, 4,728,730 (FREY) 1 MARCH 1988 SEE COLUMNS 1-2	1-77
A,P	US, A, 808,708 (YOSHIDA) 28 FEBRUARY 1989 SEE ABSTRACT.	1-77
X A	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, VOLUME 106, ISSUED 1984 (WASHINGTON, DC) WOJCIECH J. STEC ET AL., "AUTOMATED SOLID-PHASE SYNTHESIS, SEPARATION, AND STERO CHEMISTRY OF PHOSPHOROTHIOATE ANALOGUES OF OLIGODEOXY-RIBONUCLEOTIDES, PAGES 6077-6079, SEE COMPLETE DOCUMENT.	33,35,71 1-77

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04 August 1989	13 SEP 1989
International Searching Authority	**Signature of Authorized Officer**
RO/US	GARY L. KUNZ Gary L. Kunz

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FR M THE SEC ND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	TETRAHEDRON LETTERS, VOLUME 27, NO. 46, ISSUED 1986 (GREAT BRITIAN) BRIAN C. FROEHLER, "DEOXYNUCLEOSIDE H-PHOSPHONATE DIESTER INTERMEDIATES IN THE SYNTHESIS OF INTERNUCLEOTIDE PHOSPHATE ANALOGUES," PAGES 5575 TO 5578, SEE PAGE 5575.	47-77
Y	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, VOLUME 92, NO. 15, ISSUED 1970 (WASHINGTON D.C.), F. ECKSTEIN, "NUCLEOSIDE PHOSPHO-THIOATES," PAGES 4718 TO 4723, SEE STRUCTURES 11-13.	1-46
A	US, A, 4,373,071 (ITAKURA) 8 FEBRUARY 1983 SEE COLUMNS 1-3.	1-77
A	US, A, 4,668,777 (CARUTHERS) 26 MAY 1987 SEE COLUMNS 1-3.	1-77
A	US, A, 4,415,732 (CARUTHERS) 15 NOVEMBER 1983	1-77

FURTHER INFORMATION CONTINUED FR M THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

(SEE ATTACHMENT)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. TELEPHONE PRACTICE

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.